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# TRANSMITTAL FORM

*(to be used for all correspondence after initial filing)*

| | |
|---|---|
| Application Number | 09/652,493 |
| Filing Date | August 31, 2000 |
| First Named Inventor | Mina J. Bissell |
| Art Unit | 1642 |
| Examiner Name | Yu |
| Attorney Docket Number | IB-1398 |

Total Number of Pages in This Submission: 109

## ENCLOSURES *(Check all that apply)*

- [X] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
- [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [X] Appeal Communication to Group **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) (please Identify below): Copies of Appeal Brief; Attachments (references & CVs); Duplicate Fee Transmittal Form; and Return Postcard

**Remarks**

Appeal brief in triplicate pursuant to 37 C.F.R. 1.192 (12 pages x 3);

Attachments (4) - 23 pages in triplicate.

Fee Transmittal Sheet submitted in duplicate for payment by deposit account 120690 (1 page x 2).

## SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

| | |
|---|---|
| Firm or Individual name | David J. Aston; Lawrence Berkeley National Laboratory |
| Signature | |
| Date | 2-10-04 |

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| Typed or printed name | Sharon Kay Reineman | | |
| Signature | Sharon Kay Reineman | Date | Feb. 10, 2004 |

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# FEE TRANSMITTAL for FY 2004

*Effective 10/01/2003. Patent fees are subject to annual revision.*

[X] Applicant claims small entity status. See 37 CFR 1.27

**TOTAL AMOUNT OF PAYMENT** ($) 165.00

| *Complete if Known* | |
|---|---|
| Application Number | 09/652,493 |
| Filing Date | August 31, 2000 |
| First Named Inventor | Mina J. Bissell |
| Examiner Name | Yu |
| Art Unit | 1642 |
| Attorney Docket No. | IB-1398 |

## METHOD OF PAYMENT *(check all that apply)*

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## FEE CALCULATION

### 1. BASIC FILING FEE

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description | Fee Paid |
|---|---|---|---|---|---|
| 1001 | 770 | 2001 | 385 | Utility filing fee | |
| 1002 | 340 | 2002 | 170 | Design filing fee | |
| 1003 | 530 | 2003 | 265 | Plant filing fee | |
| 1004 | 770 | 2004 | 385 | Reissue filing fee | |
| 1005 | 160 | 2005 | 80 | Provisional filing fee | |

SUBTOTAL (1) ($)

### 2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

| | Extra Claims | | Fee from below | | Fee Paid |
|---|---|---|---|---|---|
| Total Claims | | -20** = | | X | = |
| Independent Claims | | - 3** = | | X | = |
| Multiple Dependent | | | | | = |

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description |
|---|---|---|---|---|
| 1202 | 18 | 2202 | 9 | Claims in excess of 20 |
| 1201 | 86 | 2201 | 43 | Independent claims in excess of 3 |
| 1203 | 290 | 2203 | 145 | Multiple dependent claim, if not paid |
| 1204 | 86 | 2204 | 43 | ** Reissue independent claims over original patent |
| 1205 | 18 | 2205 | 9 | ** Reissue claims in excess of 20 and over original patent |

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

### 3. ADDITIONAL FEES

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description | Fee Paid |
|---|---|---|---|---|---|
| 1051 | 130 | 2051 | 65 | Surcharge - late filing fee or oath | |
| 1052 | 50 | 2052 | 25 | Surcharge - late provisional filing fee or cover sheet | |
| 1053 | 130 | 1053 | 130 | Non-English specification | |
| 1812 | 2,520 | 1812 | 2,520 | For filing a request for *ex parte* reexamination | |
| 1804 | 920* | 1804 | 920* | Requesting publication of SIR prior to Examiner action | |
| 1805 | 1,840* | 1805 | 1,840* | Requesting publication of SIR after Examiner action | |
| 1251 | 110 | 2251 | 55 | Extension for reply within first month | |
| 1252 | 420 | 2252 | 210 | Extension for reply within second month | |
| 1253 | 950 | 2253 | 475 | Extension for reply within third month | |
| 1254 | 1,480 | 2254 | 740 | Extension for reply within fourth month | |
| 1255 | 2,010 | 2255 | 1,005 | Extension for reply within fifth month | |
| 1401 | 330 | 2401 | 165 | Notice of Appeal | |
| 1402 | 330 | 2402 | 165 | Filing brief in support of an appeal | 165.00 |
| 1403 | 290 | 2403 | 145 | Request for oral hearing | |
| 1451 | 1,510 | 1451 | 1,510 | Petition to institute a public use proceeding | |
| 1452 | 110 | 2452 | 55 | Petition to revive - unavoidable | |
| 1453 | 1,330 | 2453 | 665 | Petition to revive - unintentional | |
| 1501 | 1,330 | 2501 | 665 | Utility issue fee (or reissue) | |
| 1502 | 480 | 2502 | 240 | Design issue fee | |
| 1503 | 640 | 2503 | 320 | Plant issue fee | |
| 1460 | 130 | 1460 | 130 | Petitions to the Commissioner | |
| 1807 | 50 | 1807 | 50 | Processing fee under 37 CFR 1.17(q) | |
| 1806 | 180 | 1806 | 180 | Submission of Information Disclosure Stmt | |
| 8021 | 40 | 8021 | 40 | Recording each patent assignment per property (times number of properties) | |
| 1809 | 770 | 2809 | 385 | Filing a submission after final rejection (37 CFR 1.129(a)) | |
| 1810 | 770 | 2810 | 385 | For each additional invention to be examined (37 CFR 1.129(b)) | |
| 1801 | 770 | 2801 | 385 | Request for Continued Examination (RCE) | |
| 1802 | 900 | 1802 | 900 | Request for expedited examination of a design application | |

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 165.00

## SUBMITTED BY

(Complete *(if applicable)*)

| | | | | | |
|---|---|---|---|---|---|
| Name *(Print/Type)* | David J. Aston | Registration No. *(Attorney/Agent)* | 28,051 | Telephone | 510-486-7058 |
| Signature | David J. Aston | | | Date | Feb 10 '04 |

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Customer Number
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bissell et al.

| | | | |
|---|---|---|---|
| Serial No.: | 09/652,493 | Group No.: | 1642 |
| Filed: | August 31, 2000 | Examiner: | Yu |

Entitled: DESIGN OF NOVEL DRUG SCREENS BASED ON THE NEWLY FOUND ROLE OF DYSTROGLYCAN PROTEOLYSIS IN TUMOR CELL GROWTH

# APPEAL BRIEF UNDER 37 CFR 1.192

Commissioner for Patents
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Alexandria, VA 22313-1450



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Dated: Feb. 10, 2004 By: Shawn Kay Reineman

Dear Sir:

Applicant hereby submits this brief in support of an appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. This brief is filed pursuant to Applicants' Notice of Appeal filed with the USPTO by facsimile transmission on December 19, 2003. Appropriate transmittal and fee papers are transmitted herewith.

CONTENTS Page



## 1. Real Party in Interest

The real parties in interest are The Regents of the University of California and the United States Government.

## 2. Related Appeals and Interferences

There are no known related appeals (or interferences).

## 3. Status of Claims

Pursuant to Final Office Action dated July 24, 2003, claims 1-8, 22-24, 29 and 30 are pending in the application, and these claims are all finally rejected. Claims 9-21 and 25 were earlier cancelled without prejudice pursuant to a restriction requirement. Claims 29 and 30 were added by amendment dated August 15, 2002.

## 4. Status of Amendments

All Amendments have been entered.

## 5. Summary of Invention

The present invention is directed to the finding by applicants that a previously unrecognized fragment of α-dystroglycan, having an Mr of 120-130kD, is shed from cells having tumorigenic properties.

Dystroglycan was originally identified in skeletal muscle as a component of the dystrophin-glycoprotein complex. It is composed of α- and β-subunits which are encoded by a single gene, and cleaved into two proteins by posttranslational processing. Dystroglycan is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to a transmembrane glycoprotein, β-dystroglycan. Normal α dystroglycan has a molecular weight of about 180 kD. Dystroglycan spans the sarcolemma (muscle fiber membrane) and its known function is to provide a connection between the extracellular matrix and the cytoskeleton. Dystrophin deficiency causes a drastic reduction of the dystroglycan complex in the sarcolemma and, thus, loss of linkage between the subsarcolemmal cytoskeleton and the extracellular matrix, eventually leading to muscle cell death in Duchenne muscular dystrophy.

The present application extends previous research from the laboratory of the present inventors, as illustrated in Attachment 1, Lelievre et al. "Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus," Proc. Acad. Sci. 95:14711-14716 (Dec. 1998). That is, investigations have shown certain links between tumorgenic phenotypes and properties

on the *outside* of cells, rather than genetic changes on the inside of cells. The present work is consistent with that theme in that the conventional role of dystroglycan involves interaction with the extracellular matrix, rather than a role in growth control. Specifically, the present work is directed to a newly found behavior of α dystroglycan in cancer cells. Fig. 1 illustrates this by showing an SCg6 mammary carcinoma cell supernatant having a distinct band in the 120-130kD region, representing a shed α-dystroglycan fragment. (SCg6 is known to be a tumorigenic cell line. See Attachment 2.). To detect the present 120-130kD fragment, one may use a cell medium assay (using the disclosed antibody IIH6 on p. 16 of the Specification) or use a cellular assay to determine the existence and extent of this shedding. The degree to which the α-dystroglycan on a cell surface has been cleaved and shed into the medium correlates with the tumorigenicity of the cell. In Fig. 2, it is shown that 5 of 8 tumor cell lines tested lacked detectable cell surface α-dystroglycan.

Accordingly, the present claims are directed to methods of detecting the shedding of the 120-130 kD fragment.

**6. Issues**

The presently outstanding final office action sets forth one rejection, raising the following issues:

A. Are Claims 1-8, 22-23, 29 and 30 enabled by the specification?

B. Does Dr. Campisi's Declaration constitute objective evidence that the specification contains sufficient guidance to enable one of ordinary skill in the art to make and use the claimed invention(s)?

C. Is undue experimentation required for assessing whether detection of α dystroglycan fragments correlates with tumor growth and tumorigenicity because it requires analysis of a large quantity of clinical samples to determine whether or not shedding of said fragments occurs at all in vivo and detection of said fragments is indicative of tumor growth and potential tumorigenicity? Since a large number of clinical samples is required, is it proper to require Applicants to submit "evidence showing that detection of α dystroglycan fragment in blood is correlated with tumor growth and/or potential tumorigenicity in vivo in order to obviate this rejection"? (See Final Office Action, page 3, end of first paragraph.)

**7. Grouping of Claims**

Insofar as the rejection is directed to a lack of "objective evidence" that shedding of the present α dystroglycan fragment is correlated to tumorigenicity all of the claims on appeal stand or fall together because they speak to shedding of the fragment in vivo. Claim 1 contains the phrase "whereby the presence of the fragment indicates higher potential tumorigenicity." Claim 22 contains the phrase "positively correlated with tumor cell growth." For purposes of the present appeal, these phrases are believed to raise the same issue.

**8. Arguments**

**A. The claims are enabled.**

Firstly, the question of enablement is directed to whether or not undue experimentation is required to make and use the claimed invention. There is no doubt in

this case that one of ordinary skill can make the presently claimed invention. What is in dispute is whether or not the use of the invention will have the beneficial result referred to in the claims, i.e. assessment of tumorigenic potential. The present method may not be a fully developed method immediately suitable for clinical use, but it has sufficient utility to meet the requirements of 35 USC 101. The Examiner doubts the assertions made throughout the specification by the inventors, both Ph.D. scientists doing full time research in this field. See inventors' CV's attachment 3 and attachment 4.

As stated in MPEP 2164.04,

> In order to make a[n enablement] rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, sought to 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, **unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.** Assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370. (emphasis supplied in bold).

In this case, no reason to doubt Applicants' assertions has been presented. Arguments that there may be cases where normal cells shed the α-dystroglycan fragment are pure speculation.

**B. The Declaration of Dr. Judith Campisi provides objective evidence that the claims are enabled.**

Dr. Judith Campisi is a Senior Staff Scientist at Lawrence Berkeley National Laboratory. She has no interest in the present patent application other than common employment with the present inventors. In her declaration of May 5, 2003, she states:

> 5. In my experience, a number of *in vitro* cell culture models are generally recognized in the art as correlating to *in vivo* conditions of tumorigenicity or tumorigenic potential. The three dimensional basement membrane assay, employed in the present application, is especially well regarded as predictive of *in vivo* cell growth behavior of tumor cells. Furthermore, the present specification describes nude mouse experiments that further confirm the correlation between the present *in vitro* assay and *in vivo* results. The present specification provides working examples describing the detection of the 120-130 kD α dystroglycan fragment in cell culture medium. It further provides results from a reasonable number of cell lines to show the correlation between shedding of this fragment *in vitro* and potential tumorigenicity, as represented in Fig. 2 of the specification. In this particular case, I believe that it is scientifically credible and plausible to extrapolate the detection of a shed dystroglycan fragment found in cell culture medium to the ability to find that same fragment, using similar techniques, in the blood or other tissue of a living animal, including a human. Furthermore, the teachings of the present specification support this expectation. I have reviewed the inventors' follow up paper, Muschler et al., "A Role for Dystroglycan in Epithelial Polarization: Loss of Function in Breast Tumor Cells," *Cancer Research* 62:7102-7109 (Dec. 2001), and note that they have obtained *in vivo* data in nude mice that correlate with their *in vitro* work. The nude mouse model is generally accepted as a model predictive of human tumor cell behavior.
>
> 6. I have reviewed the inventor's conclusions in the specification, in particular the discussion of the shedding of α dystroglycan fragments in hyperplasia and tumor cell growth. Specifically, I have read the following:

"Because α and β dystroglycan are translated as a single polypeptide, it was surprising that α dystroglycan was not detected on the cell surface of many cells when β dystroglycan was present. We concluded that, by some mechanism, α dystroglycan was being shed from the cell surface." (Page 11, first paragraph).

"We believe α dystroglycan shedding occurs principally in cells that are reorganizing and growing. Little of such activity occurs in adult tissues, except in cases like the normal processes of mammary gland development, and perhaps angiogenesis. However, such activity would occur on a large scale during hyperplasia or tumor cell growth and the accompanying angiogenesis. α Dystroglycan is shed in two forms, one which binds laminin and a smaller portion with no known binding activity. An assay that detects α dystroglycan proteolysis would be an assay for the detection of tissue re-organization and cell growth." (Page 13-14)

7. I believe that the weight of scientific evidence favors the statements quoted in Paragraph 6, rather than raising doubt as to the truth of these statements. The reference to "tissue re-organization and cell growth" also applies also to "potential tumorigenicity."

Thus, objective evidence has been provided for the method of using the presently claimed invention.

**C. Clinical trials or other in vivo data are not required under 35 USC 112 and are not necessary to show a reasonable expectation of success in this case.**

The "how to use" prong of the utility requirement does not require in vivo data for a biological invention. In accordance with MPEP 2164.02,

The issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" as used herein refers to the relationship between in vitro or in vivo animal model assays and a disclosed or a claimed method of use. An in vitro or in vivo animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If there is no correlation, then the examples do not constitute "working examples." In this regard, the issue of "correlation" is also dependent on

> the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that in vitro data did not support in vivo applications).
>
> Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an in vitro or in vivo animal model example. A rigorous or an invariable exact correlation is not required, as stated in Cross v. Iizuka, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):
>
> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed in vitro utility and an in vivo activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (Citations omitted).

Thus the "invitation" in the Final Office Action that the Applicants supply data showing detection of the shed fragment from in vivo experiments (in order to obtain allowance of the claims) is improper.

**9. Conclusion**

The present case involves a novel finding in the relationship between the cell membrance and the extracellular matrix. Modelling of in vivo cell behavior in an in vitro extracellular matrix model is accepted in the art (See Attachment 1, and Specification, page 2, first full paragraph. Specifically, the present inventors show that cleavage of a cellular adhesion molecule is correlated with increased tumorigenic potential in tests run on eight selected cell lines. The specification describes an assay that has an immediate utility in the laboratory as studying tumorigenic potential and may be extended to clinical use without undue experimentation.

Accordingly, the rejection of claims 1-8, 22-24, 29 and 30 under 35 USC 112 should be reversed and the present case passed to issuance.

Respectfully submitted,

Date: Feb 10 '04

David J. Aston
Attorney for Applicant
Reg. No.: 28,051
Tel. No. (510) 486-7503
Lawrence Berkeley Laboratory
One Cyclotron Road
MS 90B0104
Berkeley, CA 94720

## 10. Appendix: Claims on Appeal

Claim 1. A method for measuring potential tumorigenicity of mammalian cells comprising:

a. providing a sample of medium surrounding cells, and

b. detecting the presence of a fragment of α-dystroglycan in medium, said fragment having an Mr of 120-130kD, whereby the presence of the fragment indicates higher potential tumorigenicity.

Claim 2. The method of claim 1, wherein said detecting comprises:

a. adding to said sample a material selected from the group consisting of a monoclonal antibody to α-dystroglycan and laminin, and

b. measuring the size of the α-dystroglycan fragment detected.

Claim 3. The method of claim 1, wherein said cells are human mammary epithelial cells.

Claim 4. The method of claim 1, wherein said medium is blood serum.

Claim 5. A method for measuring potential tumorigenicity of cells, comprising:

a. providing a sample of said cells, and

b. detecting the presence of α-dystroglycan on the surface of the cells, whereby the absence of α-dystroglycan indicates a higher potential for tumorigenicity.

Claim 6. The method of claim 5, wherein said detecting comprises:

a. adding to said sample a monoclonal antibody to α-dystroglycan, and

b. measuring the amount of labeled α-dystroglycan detected.

Claim 7. The method of claim 5, wherein said cells are human mammary epithelial cells.

Claim 8. The method of claim 5, wherein said detecting comprises measurement of the amount of α-dystroglycan relative to the amount of β-dystroglycan, wherein a relative decrease of α-dystroglycan indicates α-dystroglycan shedding and higher potential tumorigenicity.

Claims 9 – 21 (Withdrawn).

Claim 22. A method of assaying proteolysed α-dystroglycan fragments shed from a cell into blood serum comprising the steps of:

a. contacting a serum sample to be assayed with a labeled antibody specific for an α-dystroglycan fragment, and

b. assaying the amount of bound label, wherein said α-dystroglycan fragments bound to said labeled antibody are positively correlated with tumor cell growth.

Claim 23. The method of Claim 22, wherein the α-dystroglycan fragment is an approximately 120 kD fragment.

Claim 24. The method of Claim 22, wherein the α-dystroglycan fragment is an approximately 60 kD fragment.

Claims 25 – 28 (Withdrawn).

Claim 29. The method of claim 22, wherein said cell is an epithelial cell.

Claim 30. The method of claim 29, wherein said epithelial cell is a breast epithelial cell.

*Proc. Natl. Acad. Sci. USA*
Vol. 95, pp. 14711–14716, December 1998
Cell Biology

# Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus

SOPHIE A. LELIÈVRE*, VALERIE M. WEAVER*, JEFFREY A. NICKERSON†, CAROLYN A. LARABELL*, ANKAN BHAUMIK*, OLE W. PETERSEN‡, AND MINA J. BISSELL*§

*Lawrence Berkeley National Laboratory, Berkeley, CA 94720; †University of Massachusetts Medical School, Worcester, MA 01655; and ‡The Panum Institute, DK-2200 Copenhagen N, Denmark

*Communicated by Sheldon Penman, Massachusetts Institute of Technology, Cambridge, MA, September 30, 1998 (received for review August 14, 1998)*

ABSTRACT **What determines the nuclear organization within a cell and whether this organization itself can impose cellular function within a tissue remains unknown. To explore the relationship between nuclear organization and tissue architecture and function, we used a model of human mammary epithelial cell acinar morphogenesis. When cultured within a reconstituted basement membrane (rBM), HMT-3522 cells form polarized and growth-arrested tissue-like acini with a central lumen and deposit an endogenous BM. We show that rBM-induced morphogenesis is accompanied by relocalization of the nuclear matrix proteins NuMA, splicing factor SRm160, and cell cycle regulator Rb. These proteins had distinct distribution patterns specific for proliferation, growth arrest, and acini formation, whereas the distribution of the nuclear lamina protein, lamin B, remained unchanged. NuMA relocalized to foci, which coalesced into larger assemblies as morphogenesis progressed. Perturbation of histone acetylation in the acini by trichostatin A treatment altered chromatin structure, disrupted NuMA foci, and induced cell proliferation. Moreover, treatment of transiently permeabilized acini with a NuMA antibody led to the disruption of NuMA foci, alteration of histone acetylation, activation of metalloproteases, and breakdown of the endogenous BM. These results experimentally demonstrate a dynamic interaction between the extracellular matrix, nuclear organization, and tissue phenotype. They further show that rather than passively reflecting changes in gene expression, nuclear organization itself can modulate the cellular and tissue phenotype.**

The cell nucleus is organized by a nonchromatin internal structure referred to as the nuclear matrix (NM; refs. 1–3). Identified NM components include coiled-coil proteins (4), cell cycle regulators (5), tissue-specific transcription factors (6, 7), and RNA splicing factors (for review see ref. 2). Although splicing factors have been shown to redistribute during cellular differentiation (8, 9) and following the induction of gene expression (10), spatial distribution of nuclear components are thought to be the consequence of changes in gene expression (8, 10, 11). However, whether NM composition and structure may themselves affect gene expression and cellular function has not been examined.

To systematically study the effect of cell growth and tissue differentiation on nuclear organization, we used a reconstituted basement membrane (rBM)-directed model of mammary gland morphogenesis (12). The HMT-3522 human mammary epithelial cells (HMECs) were isolated from reduction mammoplasty and became immortalized in culture (13). When embedded within a rBM, these cells arrest growth, organize an endogenous BM, and form polarized acinus-like structures with vectorial secretion of sialomucin into a central lumen (12). We used this model to compare the nuclear organization of HMECs cultured on a plastic surface [two-dimensional (2D) monolayer] vs. a three-dimensional (3D) rBM. Nuclear organization was assessed by examining the distribution of the coiled-coil NM proteins lamin B (14) and NuMA (15), the cell cycle regulator Rb (p110Rb; ref. 5), and the splicing factor SRm160 (formerly known as B1C8; ref. 16). These proteins had distinct spatial distribution patterns specific for proliferation, growth arrest, and acini formation. Moreover, disruption of nuclear organization in acini by either perturbing histone acetylation or directly modifying the distribution of NM proteins altered the acinar phenotype.

We previously hypothesized (17) and thereafter provided evidence that the extracellular matrix (ECM) directs morphogenesis and gene expression in mammary epithelial cells (12, 18, 19). Here we show that a reciprocal relationship exists between the ECM and nuclear organization. These findings underscore a role for nuclear organization in regulation of gene expression and provide a possible framework for how cell–ECM interactions determine cell and tissue phenotype.

## MATERIALS AND METHODS

**Cell Culture.** HMT-3522 HMECs (S-1 passage-50 cells; ref. 13) were propagated in 2D cultures in chemically defined medium (12), and growth arrest was induced by removing epidermal growth factor (EGF) for 48 hr. Cultures were prepared by embedding single cells ($8.5 \times 10^5$ cells per ml of matrix) in rBM (Matrigel, Collaborative Research) or collagen-I matrix (Cellagen AC-5, ICN) in 4-well chamber slides (Nalge). These cultures were grown for 5–10 days. Growth arrest and morphogenesis were routinely observed by days 7–9.

**Antibodies and Inhibitors.** For Western blots and/or immunostaining, we used mAbs against type IV collagen (clone CIV, Dako), β-catenin (clone 14, Transduction Laboratories, Lexington, KY), SRm160 splicing factor (clone B1C8, 16), lamin B (clone 101-B7, Matritech, Cambridge, MA), NuMA (clone 204–41, Matritech, and clone B1C11, a gift from S. Penman, Massachusetts Institute of Technology, Cambridge, MA), and polyclonal antibodies (pAbs) against Ki-67 (Novo-Castra, Newcastle, U.K.), acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY), and p110Rb (Santa Cruz Biotechnology). For bioperturbation assays, we used mAbs against lamins A/C (clone 636, Novocastra, Newcastle, U.K.)

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Abbreviations: NM, nuclear matrix; BM, basement membrane; rBM, reconstituted BM; HMEC, human mammary epithelial cells; 2D and 3D, two and three dimensional; Rb, retinoblastoma protein; ECM, extracellular matrix; EGF, epidermal growth factor.

§To whom reprint requests should be addressed at: Lawrence Berkeley National Laboratory, Life Sciences Division, MS 83-101, Berkeley, CA 94720. e-mail: mjbissell@lbl.gov.

and NuMA (clone 22, Transduction Laboratories, Lexington, KY), in addition to B1C11 and 101-B7. Trichostatin A (Wako Chemicals, Richmond, VA) was used as an inhibitor of histone deacetylase (40 nM).

**Indirect Immunofluorescence.** Cells were permeabilized *in situ* (0.5% Triton X-100 in 100 mM NaCl/300 mM sucrose/10 mM Pipes, pH 6.8/5 mM MgCl2 containing 1 mM Pefabloc Sc (AEBSF) (Boehringer Manheim)/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml trypsin inhibitor type II/250 μM NaF), fixed in 2% paraformaldehyde, and immunostained as described (18). Human mammary tissue was snap-frozen in n-hexane and embedded in Tissue-Tek O.C.T. compound (Sakura Firetek, Torrance, CA); 5-μm sections were fixed in methanol and immunostained in accordance with human protocol (KF) 01–216/93 in the laboratory of O.W.P.

**Image Acquisition, Processing, and Data Analysis.** Samples were analyzed by using a Bio-Rad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 200 microscope. Fluorescence specificity was verified by sequential fluorophore excitation. NuMA foci were analyzed by using IMAGE SPACE-3D analysis program (Molecular Probes) and normalized to 3D rBM cluster-cell number by highlighting and counting each nucleus using IMAGE SPACE-MEASURE 2D. The voxel threshold was set at 0.2 μm.

**Immunoblot Analysis.** Total cell extracts (2% SDS in phosphate-buffered saline, pH 7.4, containing 1 mM Pefabloc/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml trypsin inhibitor type II/250 μM NaF) were prepared *in situ* for 2D cultures or from acini isolated from 3D cultures by dispase treatment (5,000 units per ml caseinolytic activity, Collaborative Research). Equal amounts of protein were separated and immunoblotted as described (18).

***In Situ* NM Preparation.** *In situ* NM preparation was as previously described (20), except that 0.05% Triton X-100 and micrococcal nuclease (5 units per ml; Sigma) were used.

**Antibody-Mediated Perturbation of Nuclear Organization.** rBM-induced acini (day 10) were permeabilized for less than 2 min *in situ* (0.01% digitonin in 25 mM Hepes, pH 7.2/78 mM potassium acetate/3 mM magnesium acetate/1 mM EGTA/300 mM sucrose/1% RIA-grade BSA), rinsed twice in digitonin-free buffer, and incubated in medium containing dialyzed specific or mock mAbs (15 μg/ml) for 48 hr, after which the cells were incubated with fresh medium for an additional 48 hr. Antibody concentrations and incubation times were determined empirically. Trypan blue dye-exclusion tests and apoptosis studies verified the absence of digitonin toxicity.

## RESULTS

**Internal Nuclear Organization Is Remodeled When HMECs Are Cultured Within a Basement Membrane.** HMT-3522 HMECs, like primary HMECs, undergo morphogenesis to form tissue-like acini when cultured in a 3D rBM (12, 18). Neither cell type undergoes acinar differentiation when cultured as 2D monolayers. In proliferating 2D cultures, NuMA was diffusely distributed in the nucleus (Fig. 1*b*) except when localized to the spindle poles in mitotic cells (15), and splicing factor SRm160 was distributed into numerous speckles of heterogenous sizes (Fig. 1*c*; ref. 16). In rBM-induced acini, NuMA was redistributed into an average of eight nuclear foci (ranging from 1 to 1.6 μm in diameter) surrounded by diffusely localized NuMA protein (Fig. 1*e*), and SRm160 was distributed into an average of seven large speckles (Fig. 1*f*). In contrast, lamin B maintained a peripheral ring-like distribution around the nucleus, with some internal localization, regardless of culture conditions (Fig. 1 *a* and *d*). The distribution pattern of these proteins was conserved in NM preparations *in situ*, where chromatin was removed before immunolocalization (staining is shown for 3D rBM cultures only (Fig. 1 *g–i*).

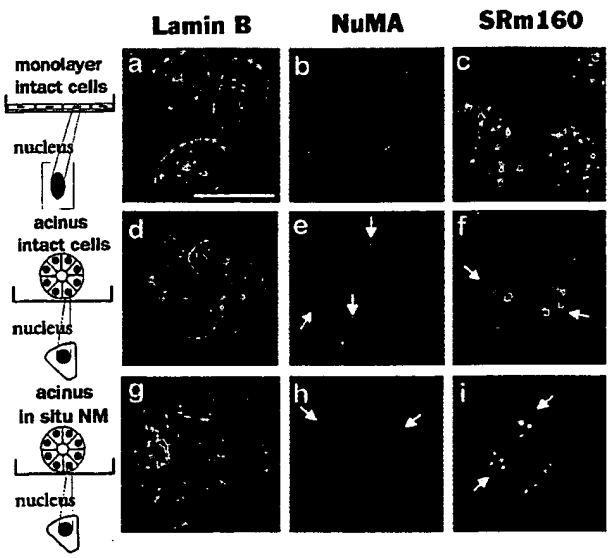



FIG. 1. NM protein redistribution in HMECs after 3D rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2-μm optical sections) of lamin B, NuMA, and splicing factor SRm160 in cells grown as 2D monolayers (*a–c*) and within 3D rBMs (*d–i*). NuMA was diffusely distributed in the nuclei of cells grown as monolayers (*b*), but reorganized into large nuclear foci in cells induced to undergo morphogenesis (acini formation) in response to a rBM (*e*). SRm160 was distributed as multiple nuclear speckles in cells cultured as monolayer (*c*), whereas it was concentrated into fewer and larger speckles in the acini (*f*). Lamin B, in contrast, consistently localized to the nuclear periphery and within intranuclear patches (*a* and *d*). The distribution of lamin B (*g*), NuMA (*h*), and SRm160 (*i*) after *in situ* NM preparation of cells cultured in 3D rBM was similar to that observed in intact cells (*d–f*). Arrows indicate nuclei found within the plane of the section. (Bar = 10 μm.)

We next examined NuMA and SRm160 distribution at different stages of 3D rBM-induced morphogenesis. After embedment in rBM, cells proliferated to form small clusters by days 3–5 but lacked β-catenin at cell–cell junctions, and collagen IV staining was discontinuous (Fig. 2 *Aa–Ac*). After growth arrest (days 6–10), cells assembled a continuous endogenous BM and formed polarized acinus-like structures with organized adherens junctions (Fig. 2 *Ad–Af*). NuMA was uniformly distributed in the nuclei of proliferating cells (Fig. 2*B*a), but became concentrated into distinct foci of differing sizes after growth arrest (day 7; Fig. 2*Bb*), and into larger and fewer foci on completion of morphogenesis (day 10, Fig. 2*Bc*). NuMA and the splicing factor SRm160 were not colocalized in proliferating cells (Fig. 2 *Ba'* and *Ba''*), but NuMA foci and SRm160 speckles were closer together after growth arrest (Fig. 2 *Bb'* and *Bb''*) and were completely colocalized in large assemblies after the completion of morphogenesis (Fig. 2*B*, c' and c''). These spatial changes in NuMA arrangement occurred without significant modifications in the level of NuMA expression or molecular weight, as determined by using Western blot analysis (Fig. 2*Be*). These experiments demonstrate that specific NM proteins undergo spatial rearrangement during rBM-induced acinar morphogenesis. Because the existence of NuMA in differentiated tissue has been questioned (21), we studied NuMA in the normal resting human mammary gland. Intense staining was observed in the epithelial cells of acini and ducts, where NuMA was distributed in foci of different sizes and resembled the acinar stages recapitulated in 3D rBM cultures (Fig. 2*Bd*).

**Growth Arrest Is Associated With Changes in NuMA and Rb Distribution.** ECM-directed growth arrest is an early and critical step in mammary epithelial cell morphogenesis (12). To distinguish between the effect of ECM-directed growth arrest

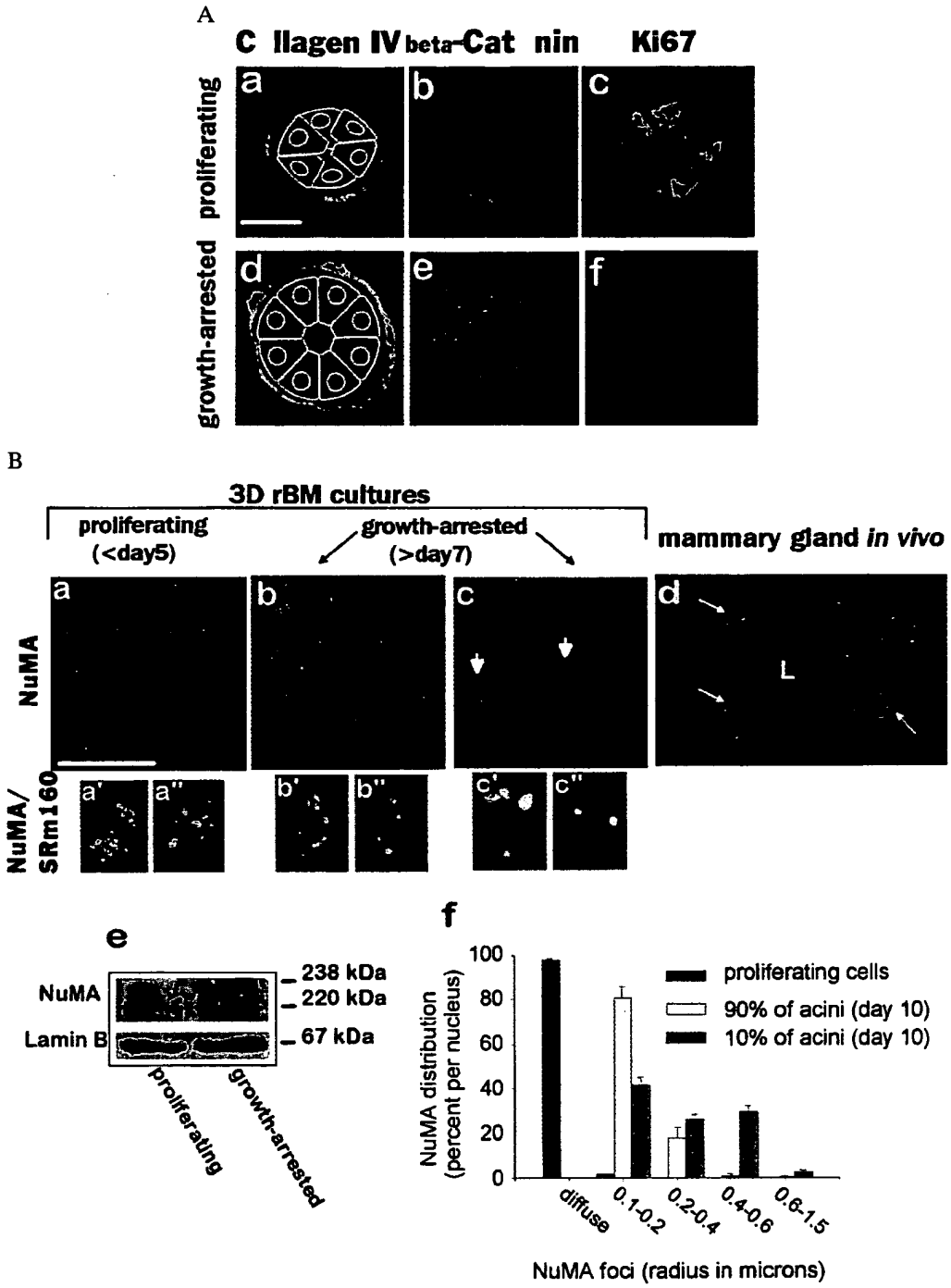



FIG. 2. (*A*) Distribution of structural proteins during rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2-μm optical sections) of collagen IV, β-catenin, and Ki-67 in HMECs embedded within a rBM for 3–4 days (proliferating cells; *a–c*), and for 7–10 days (growth-arrested acini; *d–f*). Coincident with growth arrest and acinar morphogenesis, HMECs deposited an organized endogenous collagen IV-rich BM (*a* vs. *d*), whereas β-catenin relocalized from the cytosol and basal plasma membrane to sites of cell–cell adhesion (*b* vs. *e*). Acinar morphogenesis was associated with cell cycle exit, as indicated by the loss of Ki-67 staining (*c* vs. *f*). (*B*). Spatial analysis of NuMA and splicing factor SRm160 redistribution during rBM-induced acinar morphogenesis. Confocal Texas red fluorescence images (0.2-μm optical sections) of NuMA (*a–c*) and double-labeled NuMA (Texas red), and fluorescein isothiocyanate (FITC) green-stained SRm160 (*a'*, *a''*, *b'*, *b''*, *c'*, and *c''*) in HMT-3522 cells proliferating (a, a', and a'') and undergoing morphogenesis (*b*, *b'*, *b''*, *c*, *c'*, and *c''*) in response to a rBM. In proliferating cells, NuMA was diffusely distributed (*a*) and did not colocalize with SRm160 (*a'* and *a''*). After growth arrest, NuMA coalesced into foci of increasing size (0.2–2 μm; *f*) in association with the establishment of mature tissue-like structures (acini; *b* and *c*) Nine nuclei are shown in *b*. Only the larger NuMA foci observed in late morphogenesis fully colocalized with SRm160 (*b'*, *b''*, *c'*, and *c''*). (*d*) In the ductal and acinar HMECs of the mammary gland, *in vivo*, NuMA was localized in foci with a size distribution comparable to that observed in most of the HMEC nuclei of differentiating rBM cultures shown in *b*. (*e*) Western blot analysis of NuMA and Lamin B showed no difference in protein expression or size between proliferating and growth-arrested HMECs grown within rBMs. Arrows indicate nuclei. (Bars = 10 μm.)

and changes caused by tissue structure and polarity, the localization of NuMA and SRm160 was compared between growth-arrested and proliferating cells cultured in monolayers. Less than 5% of the cells remained in the cell cycle after

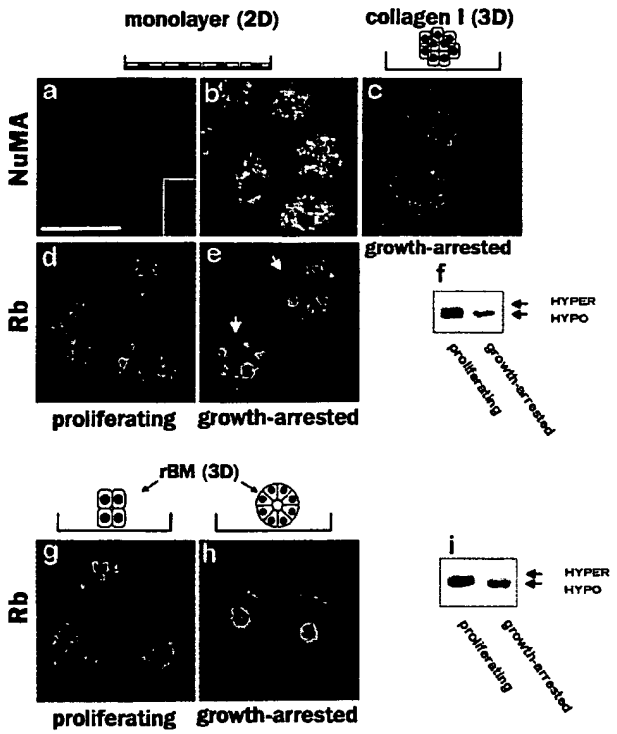



FIG. 3. Effect of growth status on the distribution of NM proteins. Confocal fluorescence images (0.2-μm optical sections) of Texas red-stained NuMA (*a–c*) and fluorescein isothiocyanate (FITC) green-stained Rb (*d*, *e*, *g*, and *h*) in cells proliferating as 2D monolayers (*a* and *d*) and within 3D rBMs (*g*) and cells growth-arrested in monolayer (*b* and *e*) and within collagen-I (*c*) or a rBM (*h*). NuMA was diffusely distributed in the nucleus of proliferating HMECs grown as monolayers (*a*) and reorganized into random aggregates on growth arrest induced by EGF removal (*b*). The settings for image recording were the same as for *a*. Aggregates appear white because of saturation of the signal. NuMA was distributed in random aggregates or in small foci in growth-arrested and BM-free cell colonies obtained after 10 days of culture within collagen-I (*c*). Rb was diffusely distributed in the nucleus of proliferating cells grown either in monolayer (*d*) or in 3D rBM (*g*); however, on growth arrest, the protein redistributed into several foci in the monolayer propagated cells (*e*) but coalesced into a central, single nuclear focus in the rBM-induced acini (*h*); the dotted line indicates outer nuclear limit. Western blot analysis of Rb in proliferating and growth-arrested cells grown as monolayers (*f*) or within a 3D rBM (*i*) shows that the hyperphosphorylated isoform was present only in proliferating cells. Arrows indicate nuclei. (Bar = 10 μm.)

growth arrest induced by EGF removal, as indicated by the absence of detectable Ki-67 immunostaining (data not shown). NuMA was uniformly distributed in the nuclei of proliferating cells but coalesced into denser areas on growth arrest (Fig. 3 *a* and *b*). The irregular geometric quality of these dense areas was distinct from the circular foci pattern observed in growth-arrested 3D rBM-grown cells. In contrast, no significant change in the multispeckled distribution of SRm160 was detected under these conditions (data not shown). The relationship between nuclear organization and growth status was further investigated by examining the distribution of the cell cycle regulator Rb. Rb redistributed from a diffuse nuclear pattern in proliferating HMECs into a few large foci in growth-arrested cells (Fig. 3 *d* and *e*). Strikingly, the distribution of Rb in the growth-arrested 2D cultures was distinct from that observed in the growth-arrested 3D cultures (compare Fig. 3 *e* and *h*), which may reflect differences in the state of growth arrest between 2D monolayer and 3D rBM cultures. The monofocal pattern of Rb observed in 3D culture coincided with growth arrest. Western blot analysis showed that hypophosphorylated Rb was associated with the NM in 3D cultures (data not shown) as was previously reported for 2D cultures (5). Moreover, the diffuse distribution observed in proliferating cells was associated with the hyperphosphorylated form of the protein (Fig. 3 *f* and *i*).

Because growth arrest in 3D rBM precedes the final stages of acinar morphogenesis (12), we examined the relationship between the large NuMA foci and the formation of a polarized endogenous BM. HMECs cultured in a 3D collagen-I matrix form growth-arrested organized colonies but do not assemble a polarized, endogenous BM (22). Therefore, we compared NuMA distribution in cells grown in rBM to those grown in type I collagen. After 12 days in collagen I, NuMA was distributed as small foci or irregular dense aggregates (Fig. 3*c*), similar to the pattern observed in growth-arrested cells in 2D cultures. Thus, NuMA redistribution into dense areas and small foci is induced by growth arrest, but the coalescence of the foci into larger and distinct structures requires the presence of a BM.

**Cross-Modulation Between NuMA Distribution, Chromatin Structure, and the Acinar Phenotype.** The degree of histone acetylation has been shown to regulate chromatin structure and gene expression (19, 23). Histone acetylation was altered in the acini by using the histone deacetylase inhibitor trichostatin A. After 2 hr of treatment, NuMA foci began to disperse, and several cells entered the cell cycle, as measured by an increase in the Ki-67 labeling index. After 24 hr of treatment, NuMA was diffusely distributed in all nuclei (Fig. 4 *e* vs. *a*), and the acinar phenotype was altered as shown by loss of the endogenous BM (Fig. 4 *f* vs. *b*), redistribution of β-catenin (Fig. 4 *g* vs. *c*), and the presence of mitotic cells, as shown by mitotic spindle-pole staining of NuMA (Fig. 4*e*, arrow). In contrast, trichostatin A did not alter the cell phenotype or the distribution of NuMA (data not shown).

Because NuMA is essential for postmitotic nuclear assembly and participates in the loss of nuclear integrity during apoptosis (24, 25), we asked whether disruption of NuMA foci in the acini could globally influence nuclear organization and affect the acinar phenotype. Rapid and reversible digitonin permeabilization was used to load cells with either anti-NuMA mAbs or with an $IgG_1$ mock mAb. The NuMA mAb B1C11, but not an N-terminal-specific mAb (clone 22; data not shown), disrupted NuMA organization, causing the protein to become diffusely redistributed within the nucleus as revealed by the secondary Ab (Fig. 4*i*). Chromatin structure was altered, as shown by the rearrangement of acetylated histone H4 distribution (Fig. 4 *l* vs. *d*). More dramatically, disruption of NuMA organization altered the acinar phenotype, as indicated by loss of the endogenously deposited BM (Fig. 4*j*). Because the loss could be prevented by treatment with GM6001, a potent metalloprotease inhibitor (Fig. 4*n*; ref. 26), we conclude that NuMA disruption led to induction and/or activation of a metalloprotease. Similar treatment of the acini with mAbs against lamins A/C or lamin B did not induce any change in histone H4 acetylation, BM integrity, or lamin distribution, even though these Abs reached their nuclear targets, as shown by secondary Ab staining (Fig. 4*n* and data not shown).

## DISCUSSION

By modifying the cellular microenvironment, we have demonstrated that nuclear organization rearranges dramatically in HMECs after growth arrest and tissue-like acinar morphogenesis (Scheme 1). The use of the 3D-rBM culture assay has enabled us also to show that alterations of nuclear organization can modify the cellular and tissue phenotype.

Previously documented changes in nuclear organization have been broadly descriptive. By systematically analyzing the distribution of three NM proteins in 2D and 3D cultures, we have determined that precise nuclear rearrangements occur

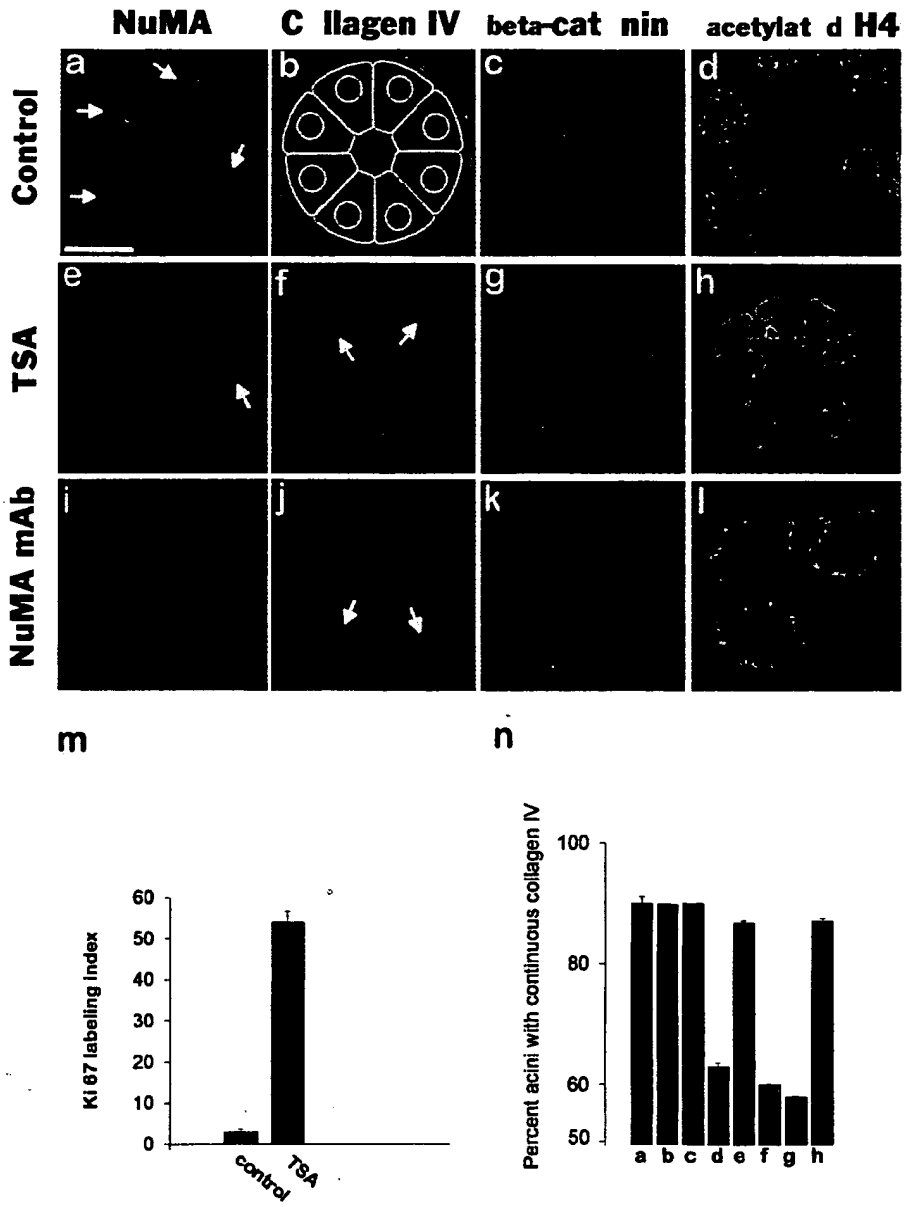
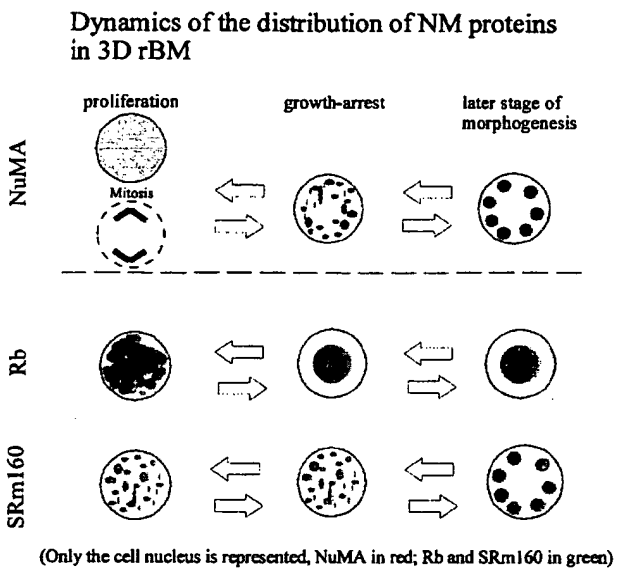



FIG. 4. Cross-modulation between chromatin structure, NM organization, and the acinar phenotype. Confocal fluorescence images (0.2-$\mu$m optical sections) of NuMA (*a*, *e*, and *i*), collagen IV (*b*, *f*, and *j*), $\beta$-catenin (*c*, *g*, and *k*), and acetylated histone H4 (*d*, *h*, and *l*) in control, trichostatin A (TSA)-treated, and NuMA mAb-incubated acini (day 10 of 3D rBM culture). (*a–d*) Nuclear organization and acinar phenotype in controls. Acini exhibit NuMA foci (*a*), an organized endogenous collagen IV-rich BM (*b*), cell–cell localized $\beta$-catenin (*c*), and dispersed acetylated H4 histone (*d*). (*e–h*) Effects of TSA on nuclear architecture and acinar phenotype. After 24 hr of TSA treatment (40 nM), >55% of the cells entered the cell cycle, as indicated by an increase in Ki-67 labeling index (*m*) and the appearance of mitotic cells (arrow in *e*). NuMA was uniformly distributed in the nuclei (*e*), collagen IV disappeared (*f*), $\beta$-catenin was released from the cell–cell interface (*g*), and the pattern of histone H4 acetylation was altered (*h*). (*i–l*) Effects of mAb-induced NuMA foci disruption on nuclear organization and acinar phenotype. Introduction of a NuMA mAb into the nuclei of the acini by using reversible digitonin permeabilization led to the disruption of NuMA foci (*i*), degradation of the collagen IV-rich BM (arrows in *j*), and the nuclear marginalization of acetylated H4 histone (*l*). There was no consistent alteration observed for $\beta$-catenin other than increased basal labeling (*k*). These effects were not observed with mock IgGs or mAbs to lamins A/C or B. (*n*) BM degradation after mAb-induced NuMA disruption in acini. Analysis of the percentage of acini with intact collagen IV-rich BMs in relation to control/digitonin-permeabilized (DP) acini (*a*), mock-IgG mAb-treated/DP acini (*b*), NuMA mAb-treated/nonpermeabilized acini (*c*), NuMA mAb-treated/DP acini (*d*), NuMA mAb-treated/DP acini + the metalloproteinase inhibitor GM6001 (*e*), NuMA mAb-treated/DP acini + the inactive metalloproteinase inhibitor GM1210 (*f*), NuMA mAb-treated/DP acini + the uPA inhibitor, aprotinin (*g*), and Lamin B mAb-treated/DP acini (*h*). Acini (>35%) degraded their endogenous BMs in response to disruption of NuMA (*d*). The BM loss could be rescued by treatment with the metalloproteinase inhibitor GM6001 (*e*), but not its inactive analogue (*f*) or a uPA protease inhibitor (*g*). (Bar = 10 $\mu$m.)

with growth arrest and after rBM-induced morphogenesis. In 3D rBM cultures, both NuMA and Rb were diffusely distributed in the nucleus of proliferating cells. After growth arrest, NuMA was relocalized into discrete foci, whereas Rb redistributed into a central nuclear mass. These patterns of distribution were different from those observed in growth-arrested cells in monolayer 2D cultures, suggesting that there may be different states of growth arrest in 2D and 3D rBM cultures (27). Because NuMA distribution in 3D collagen I cultures was comparable to that observed in growth-arrested 2D cultures, our results suggest that 3D organization of cells *per se* cannot explain the differences seen between monolayer and 3D rBM




SCHEME 1

cultures. This finding implies that BM signaling is necessary for the ultimate nuclear organization within the acini. Indeed, the presence of large and distinct NuMA foci was observed only in mature 3D rBM cultures and in adult resting mammary gland *in vivo*, where the acini were surrounded by a continuous endogenous BM. The mammary gland undergoes developmental cycles of growth and differentiation even in adults; this may account for the heterogeneity of foci size observed *in vivo* and may further explain the absence of the very large NuMA foci in subpopulations of differentiated acini (Fig. 2*Bc*). Whether the pattern of NuMA distribution indeed corresponds to different levels of differentiation *in vivo* requires further analysis.

The antibody-directed disruption of NuMA foci in the acini induced changes in the distribution pattern of acetylated histone H4, the activation of metalloprotease(s), and the loss of BM integrity. These results, as well as our observation that NuMA progressively coalesces and eventually colocalizes with enlarged splicing-factor speckles during acini differentiation, suggests that some nuclear proteins may contain the molecular information necessary for the development and/or maintenance of the acinar phenotype. Interestingly, trichostatin-induced alteration of histone acetylation in acini also led to the disruption of NuMA foci and was associated with the loss of BM and the induction of cell proliferation. Although we do not know the molecular mechanisms responsible for phenotypic alterations induced by nuclear reorganization, our experiments demonstrate also the existence of reciprocal interactions between nuclear organization, chromatin structure, and the acinar phenotype. The BM has been shown previously to be necessary for the formation and maintenance of the functional acinus (12, 28, 29). We report here that BM-induced acinar formation is associated with the distinct spatial organization of a repertoire of NM proteins and that, conversely, perturbation of nuclear organization alters the BM and influences the acinar phenotype. These results illustrate the dynamic reciprocity between the ECM and the structural organization of the nucleus, and underscore the importance of ECM–NM communication (17) in phenotypic plasticity.

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1. Berezney, R. & Coffey, D. S. (1974) *Biochem. Biophys. Res. Commun.* **60,** 1410–1417.
2. Nickerson, J. A., Blencowe, B. J. & Penman, S. (1995) *Int. Rev. Cytol.* **162A,** 67–123.
3. Nickerson, J. A., Krockmalnic, G., Van, K. M. & Penman, S. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4446–4450.
4. Odgren, P. R., Harvie, L. W. & Fey, E. G. (1996) *Proteins* **24,** 467–484.
5. Mancini, M. A., Shan, B., Nickerson, J. A., Penman, S. & Lee, W.-H. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 418–422.
6. van Wijnen, A. J., Bidwell, J. P., Fey, E. G., Penman, S., Lian, J. B., Stein, J. & Stein, G. S. (1993) *Biochemistry* **32,** 8397–8402.
7. Nardozza, T. A., Quigley, M. M. & Getzenberg, R. H. (1996) *J. Cell Biochem.* **61,** 467–477.
8. Antoniou, M., Carmo-Fonseca, M., Ferreira, J. & Lamond, A. I. (1993) *J. Cell Biol.* **123,** 1055–1068.
9. Sahlas, D. J., Milankov, K., Park, P. C. & De Boni, U. (1993) *J. Cell Sci.* **105,** 347–357.
10. Misteli, T., Caceres, J. F. & Spector, D. L. (1997) *Nature (London)* **387,** 523–527.
11. Singer, R. H & Green, M. R. (1997) *Cell* **91,** 291–294.
12. Petersen, O. W., Rønnov-Jessen L., Howlett, A. R. & Bissell, M. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9064–9068.
13. Briand, P., Petersen, O. W. & Van Deurs, B. (1987) *In Vitro Cell. Dev. Biol.* **23,** 181–188.
14. Gerace, L., Comeau, C. & Benson, M. (1984) *J. Cell Sci. Suppl.* **1,** 137–160.
15. Lydersen, B. & Pettijohn, D. (1980) *Cell* **22,** 489–499.
16. Blencowe, B. J., Issner R., Nickerson, J. A. & Sharp, P. A. (1998) *Genes Dev.* **12,** 996–1009.
17. Bissell, M. J., Hall, H. G. & Parry, G. (1982) *J. Theor. Biol.* **99,** 31–68.
18. Weaver, V. M., Petersen, O. W., Wang F., Larabell, C. A., Briand, P., Damsky, C. & Bissell, M. J. (1997) *J. Cell Biol.* **137,** 231–245.
19. Myers, C. A., Schmidhauser, C., Mellentin-Michelotti, J., Fragoso, G., Roskelley, C. D., Casperson, G., Mossi, R., Pujuguet, P., Hager G. & Bissell, M. J. (1998) *Mol. Cell. Biol.* **18,** 2184–2195.
20. He, D., Nickerson, J. A. & Penman, S. (1990) *J. Cell Biol.* **110,** 569–580.
21. Merdes, A. & Cleveland, D. W. (1998) *J. Cell Sci.* **111,** 71–79.
22. Howlett, A. R., Bailey, N., Damsky, C., Petersen, O. W. & Bissell, M. J. (1995) *J. Cell Sci.* **108,** 1945–1957.
23. Pazin, M. J. & Kadonaga, J. T. (1997) *Cell* **89,** 325–328.
24. Compton, D. A. & Cleveland, D. W. (1994) *Curr. Opin. Cell Biol.* **6,** 343–346.
25. Weaver, V. M., Carson, C. E., Walker, P. R., Chaly, N., Lach, B., Raymond, Y., Brown, D. L. & Sikorska, M. (1996) *J. Cell Sci.* **109,** 45–56.
26. Grobelny, D., Poncz, L. & Galardy, R. E. (1992) *Biochemistry* **31,** 7152–7154.
27. Dhawan, J. & Farmer, S. R. (1990) *J. Biol. Chem.* **266,** 8470–8475.
28. Streuli, C. H., Bailey, N. & Bissell, M. J. (1991) *J. Cell Biol.* **115,** 1383–1395.
29. Boudreau, N., Sympson, C. J., Werb, Z. & Bissell, M. J. (1995) *Science* **267,** 891–893.

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# A Novel Pathway for Mammary Epithelial Cell Invasion Induced by the Helix-Loop-Helix Protein Id-1

PIERRE-YVES DESPREZ,[1,2]* CLAUDIA QIAO LIN,[1,2] NICOLE THOMASSET,[1]†
CAROLYN J. SYMPSON,[1]‡ MINA J. BISSELL,[1] AND JUDITH CAMPISI[1]

*Department of Cell and Molecular Biology, Life Sciences Division, Berkeley National Laboratory, University of California, Berkeley, California 94720,[1] and Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, California 94115[2]*

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**Mammary epithelial cells undergo changes in growth, invasion, and differentiation throughout much of adulthood, and most strikingly during pregnancy, lactation, and involution. Although the pathways of milk protein expression are being elucidated, little is known, at a molecular level, about control of mammary epithelial cell phenotypes during normal tissue morphogenesis and evolution of aggressive breast cancer. We developed a murine mammary epithelial cell line, SCp2, that arrests growth and functionally differentiates in response to a basement membrane and lactogenic hormones. In these cells, expression of Id-1, an inhibitor of basic helix-loop-helix transcription factors, declines prior to differentiation, and constitutive Id-1 expression blocks differentiation. Here, we show that SCp2 cells that constitutively express Id-1 slowly invade the basement membrane but remain anchorage dependent for growth and do not form tumors in nude mice. Cells expressing Id-1 secreted a ~120-kDa gelatinase. From inhibitor studies, this gelatinase appeared to be a metalloproteinase, and it was the only metalloproteinase detectable in conditioned medium from these cells. A nontoxic inhibitor diminished the activity of this metalloproteinase in vitro and repressed the invasive phenotype of Id-1-expressing cells in culture. The implications of these findings for normal mammary-gland development and human breast cancer were investigated. A gelatinase of ~120 kDa was expressed by the mammary gland during involution, a time when Id-1 expression is high and there is extensive tissue remodeling. Moreover, high levels of Id-1 expression and the activity of a ~120-kDa gelatinase correlated with a less-differentiated and more-aggressive phenotype in human breast cancer cells. We suggest that Id-1 controls invasion by normal and neoplastic mammary epithelial cells, primarily through induction of a ~120-kDa gelatinase. This Id-1-regulated invasive phenotype could contribute to involution of the mammary gland and possibly to the development of invasive breast cancer.**

The epithelial cells of the mammary gland undergo coordinate changes in growth, differentiation, and invasion of the surrounding ECM during embryonic development and puberty, and throughout much of adulthood during each menstrual cycle. Particularly striking changes occur during pregnancy, lactation, and involution. The molecular mechanisms that control the growth and functional differentiation of mammary epithelial cells are slowly being elucidated, but far less is known about the transient invasive behavior of normal breast epithelial cells.

Normal breast epithelial cells proliferate and invade the surrounding ECM during the fetal and postnatal development of the gland, and then more vigorously at puberty as the branches of the mammary epithelial tree are formed. After puberty, there are minor waves of mammary epithelial-cell proliferation during each estrous cycle (16, 46). The most striking activity of mammary epithelial-cell proliferation and invasion occurs during pregnancy, as the gland expands in preparation for lactation (45). The proliferation and invasion of breast epithelial cells cease during late pregnancy, whereupon the cells functionally differentiate—that is, they express and secrete milk proteins (44). The epithelial cells remain proliferatively quiescent and functionally differentiated throughout lactation. At the end of lactation, the mammary gland undergoes involution, during which time there is an early and transient reactivation of epithelial-cell proliferation, followed by extensive ECM degradation and epithelial-cell death by apoptosis. The extensive remodeling of the mammary gland that occurs during involution entails the stepwise activation of several MMPs by the stromal and epithelial cells of the gland (29, 41). The involuting gland eventually returns to its prepregnancy structure.

Invasion of the ECM by normal epithelial cells must be tightly regulated and self-limiting. This control is clearly important for the mammary gland to develop and function normally. Control over normal invasive properties is also important in order to prevent neoplastic cells from invading the surrounding ECM. Most cancers develop from epithelial cells, and a hallmark of malignancy is invasion of the ECM by neoplastic epithelial cells (38). In many experimental models of tumorigenesis, an invasive phenotype develops subsequent to neoplasia and often entails expression of ECM-degrading enzymes commonly expressed by mesenchymal or stromal cells. These enzymes include the MMPs stromelysin and the 72- and 92-kDa collagenases (19, 48). It is not clear whether tumor cells express these MMPs because they are normally expressed

* Corresponding author. Mailing address: Geraldine Brush Cancer Research Institute, Stern Building, 2330 Clay St., San Francisco, CA 94115. Phone: (415) 561-1760. Fax: (415) 561-1390. E-mail: pdesprez@cooper.cpmc.org.

† Present address: INSERM Unite 433, Faculte de Medecine Laennec, 69372 Lyon, France.

‡ Present address: Searle Research and Development, Monsanto Company, Immunology Department-AA4G, North Chesterfield, MO 63198.

when epithelial cells transiently invade the ECM during normal tissue morphogenesis or because they frequently acquire mesenchymal characteristics upon transformation. It was recently shown by in situ hybridization that these MMPs are expressed by stromal fibroblasts during certain stages of ductal and alveolar mammary morphogenesis as well as during involution (29, 49).

In order to study normal and abnormal mammary epithelial-cell phenotypes, we developed a murine mammary epithelial-cell line, SCp2, whose growth and differentiation can be controlled in culture (8). SCp2 cells are an immortal line that originated from a heterogeneous cell population derived from a midpregnancy mouse mammary gland (7, 37). SCp2 cells grow well in serum on tissue culture plastic, where they express keratins and exhibit other epithelial characteristics. When serum is removed and they are given lactogenic hormones (insulin, prolactin, and hydrocortisone) and basement membrane components, SCp2 cells first arrest growth, then aggregate and form alveolar structures, and finally express high levels of several milk proteins (8, 36).

We have shown that the differentiation of SCp2 cells requires a sharp decline in the expression of the HLH protein Id-1 (9). Id genes encode a small family of proteins that prevent bHLH transcription factors from binding DNA (4). bHLH transcription factors comprise a large family of sequence-specific DNA binding proteins that activate the transcription of cell- and tissue-specific genes. bHLH proteins act as obligate dimers: they dimerize through the HLH domains and bind DNA through the composite basic domain. Id proteins contain HLH domains and therefore dimerize with bHLH proteins. However, because Id proteins lack basic domains, Id-bHLH heterodimers cannot bind DNA. Thus, Id proteins negatively regulate bHLH transcription factors. The bHLH superfamily contains both ubiquitous and lineage-specific transcription factors that direct many developmental and differentiation processes (20). Two of the four known Id proteins (Id-1 and Id-3) are nearly ubiquitously expressed, whereas the other two Id proteins (Id-2 and Id-4) have a more restricted pattern of expression (35). Thus, lineage-specific differentiation is determined by tissue-specific bHLH genes, which, in turn, are posttranslationally regulated by a small number of Id genes. Whether and how bHLH proteins participate in the differentiation of breast epithelial cells is not yet known.

Id-1 was the first Id protein to be identified (4). Since its initial discovery in myoblasts, it has been shown to be expressed by a variety of cell types and to inhibit the differentiation of myoblasts (18), several hematopoietic cell types (23, 26, 40), trophoblasts (6), and mammary epithelial cells (9). Id-1 was also found to be serum inducible in fibroblasts, where its expression is essential for progression into the S phase of the cell cycle (14). In contrast to the closely related Id-2 protein, Id-1 does not physically associate with the retinoblastoma tumor suppressor protein pRb (15, 17) but can functionally interact with a pRb-regulated pathway for entry into S phase (15).

Id-1 expression declines rapidly when SCp2 cells are induced to differentiate. As long as the cells remain in contact with a basement membrane and lactogenic hormones, Id-1 remains repressed and the cells do not proliferate, but they express milk proteins. By contrast, SCp2 cells that constitutively express Id-1 fail to differentiate, as judged by the expression of milk proteins, but nonetheless transiently arrest growth and form loose alveolar structures. After several days, cells that constitutively express Id-1 dissociate from each other and subsequently resume growth (9).

Here, we show that Id-1 expression confers upon SCp2 cells the ability to migrate and invade the basement membrane. However, cells that constitutively express Id-1 neither grow in soft agar nor form tumors in nude mice. Id-1 expression correlates strongly with expression of an apparently novel gelatinase of approximately 120 kDa, an MMP, which is also expressed during involution. The activity of this MMP was critical for the Id-1-regulated invasive phenotype. We also show that Id-1 expression correlates with the degree of differentiation and invasiveness of human breast cancer cells. The least-differentiated and most highly invasive cells express constitutively high levels of Id-1 and also secrete a 120-kDa gelatinase. Our results suggest that Id-1 is a regulator of the invasive phenotype of normal and neoplastic mammary epithelial cells and that it acts, at least in part, by controlling expression of a 120-kDa gelatinase. The invasive phenotype conferred by Id-1 is not a consequence of tumorigenic transformation, although it may be appropriated in a subset of aggressive breast cancers. Our data provide new insights into the control of breast epithelial-cell invasion and suggest that one or more bHLH transcription factors may repress the invasive phenotype in normal as well as neoplastic breast epithelial cells.

## MATERIALS AND METHODS

**Abbreviations.** AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; bHLH, basic helix-loop-helix; BSA, bovine serum albumin; DAPI, 4′,6′-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EHS, Englebreth Holm Swarm tumor; ECM, extracellular matrix; F12, Ham's F-12 medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HLH, helix-loop-helix; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-PCR.

**Cell culture.** SCp2 cells were grown in a 1:1 mixture of DMEM and F12 (DMEM-F12) containing 5% heat-inactivated FBS, insulin (5 μg/ml), and gentamicin (50 μg/ml) (growth medium) at 37°C in a humidified 5% $CO_2$ atmosphere, as previously described (8). To induce differentiation, cells were plated at $5 \times 10^4/cm^2$ atop basement membrane components in DMEM-F12 lacking serum but containing lactogenic hormones (insulin, 5 μg/ml; hydrocortisone, 1 μg/ml; prolactin, 3 μg/ml) (9). Unless otherwise indicated, cells were cultured for 5 days before analysis. Basement membrane ECM either was purified from EHS tumors by the method of Taub et al. (42) or was supplied as Matrigel from Collaborative Research.

SCp2 cells were transfected with the murine Id-1 cDNA driven by the mouse mammary tumor virus promoter as previously described (9). The transfected cells were initially pooled. Single cell-derived clones were subsequently derived by plating cells at limiting dilutions in 24-well plates. After 10 days, wells with visible colonies were trypsinized and replated onto 35-mm-diameter dishes. When nearly confluent, the cells were replated onto 100-mm-diameter dishes. The population was expanded by subculturing at a ratio of 1:4, and cells were used after 5 to 8 passages after the first 1:4 subculture.

The human breast cancer cell lines T47D, MCF-7, Hs578T, BT-549, MDA-MB-231, ZR75-1, and SKBR-3 were purchased from the American Type Culture Collection. The MDA-MB-436 cell line was originally purchased from the American Type Culture Collection and was given to us by R. Lupu (Berkeley National Laboratory). MDA-MB-435 cells were derived from the original cell line by selection in nude mice for the highly aggressive subpopulation (37a). Cells were passaged in DMEM containing 10% FBS and insulin (5 μg/ml; Sigma). For serum-free conditions, FBS was omitted from the medium.

**DNA synthesis and autoradiography.** Cells plated on coverslips were labeled with [$^3$H]methylthymidine (10 μCi/ml; 60 to 70 Ci/mmol) for 24 h, washed twice with PBS, then fixed for 5 min with a 1:1 (vol/vol) mixture of acetone and methanol at −20°C. Where indicated, cell nuclei were stained for 2 min with DAPI diluted 1:10,000 in PBS. The coverslips were air dried, coated with Kodak NTB2 emulsion (1:2 dilution), and exposed for 16 to 24 h. The coverslips were developed with D-19, fixed with Kodak Rapid-Fix, and viewed by phase-contrast microscopy.

**Boyden chamber invasion assays.** Invasion assays were performed in modified Boyden chambers with 8-μm-pore-size filter inserts for 24-well plates (Collaborative Research). Filters were coated with 10 to 12 μl of ice-cold basement membrane ECM at 8 to 12 mg of protein/ml. Cells ($0.5 \times 10^5$ to $1 \times 10^5$) were added to the upper chamber in 200 μl of DMEM-F12. The lower chamber was filled with 300 μl of NIH 3T3 cell-conditioned medium. Where indicated, GM6001 was added at 0.2 mM to both chambers immediately after cell plating. After a 16- to 20-h incubation, the cells were fixed with 2.5% glutaraldehyde in PBS and stained with 0.5% toluidine blue in 2% $Na_2CO_3$. Cells that remained in the basement membrane or attached to the upper side of the filter were removed

with paper towels. Cells on the lower side of the filter were examined by light microscopy and counted.

**Anchorage-dependent growth assays.** Liquefied 2% agarose was mixed with an equal volume of 2× DMEM-F12 growth medium lacking serum and supplemented with insulin (10 μg/ml) and gentamicin (100 μg/ml) (2× medium). One milliliter of the mixture was layered onto 35-mm-diameter dishes to create a 1% agarose base. Liquefied 0.6% agarose was mixed with an equal volume of 2× medium, and 10 ml of this solution was mixed with 1 ml of growth medium containing $10^5$ cells to yield $10^4$ cells/ml in 0.27% agarose; 1 ml of this cell suspension was layered on top of the 1% agarose base, and 1 ml of DMEM-F12 containing 5% FBS was added. The cells were incubated for 14 days, after which representative fields were photographed under phase-contrast microscopy.

**Tumorigenicity assays.** Cells were injected subcutaneously into nude mice at $4 \times 10^6$ cells per site, two sites per animal, and two animals for each cell type (TCL1, SCg6, SCp2, SCp2–antisense Id-1, and SCp2–Id-1). Animals injected with TCL1 and SCg6 cells developed easily detectable tumors (at least 1 $cm^3$) within 3 weeks and were sacrificed after 4 weeks. The remaining animals remained tumor negative for a minimum of 5 months.

**Immunofluorescence.** Cells cultured on coverslips were washed with PBS, fixed for 5 min with acetone-methanol (1:1, vol/vol) at −20°C, permeabilized for 5 min with 1% Triton X-100 in PBS, and washed with PBS. A rabbit polyclonal antiserum raised against bovine keratins (Dako, Carpinteria, Calif.) was diluted 1:10 in 0.2% BSA in PBS and applied for 60 min at 37°C, followed by three washes in PBS. The coverslips were then incubated with biotin-conjugated anti-rabbit antibody (1:100 dilution; Amersham Corp.) for 30 min at 37°C and were washed three times in PBS. Finally, the coverslips were incubated with fluorescein isothiocyanate-conjugated streptavidin (1:100 dilution; Amersham Corp.) for 30 min at 37°C and were washed in PBS. Cell nuclei were stained with DAPI, as described above, and the coverslips were mounted in glycerol-gelatin (Sigma) for viewing by epifluorescence.

**RNA isolation and analysis.** Total cellular RNA was isolated and purified as described by Chomczynski and Sacchi (5). The RNA (10 μg) was size fractionated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N from Amersham Corp.). The membrane was hybridized to $^{32}$P-labeled probes prepared by random oligonucleotide priming, washed, and exposed to XAR-5 film for autoradiography as described by Maniatis et al. (31). The β-casein probe was the 540-bp mouse cDNA (from J. Rosen, Baylor College of Medicine, Houston, Tex.), and the Id-1 probe was either the murine Id-1 cDNA (4) or the human Id-1 cDNA (14).

**RT-PCR and Southern analysis.** Transcripts for murine gelatinases A (72-kDa MMP) and B (92-kDa MMP) were detected by RT-PCR. cDNA was synthesized from total RNA by using SuperscriptII Reverse TranscriptaseII (Gibco-BRL), and 100 ng was used for PCR. The 5′ and 3′ PCR primers were TTGAGAAG GATGGCAAGTATGG and ACACCTTGCCATCGTTGC for gelatinase A, GGCGTGTCTGGAGATTCGA and AGGGTCCACCTTGGTCACC for gelatinase B, and ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGC TGTA for GAPDH. PCR was performed in 20 mM Tris-HCl (pH 8.8)–2 mM $MgSO_4$–10 mM KCl–10 mM $(NH_4)_2SO_4$–0.1% Triton X-100–100 μg of BSA/ml–0.125 mM deoxynucleoside triphosphates–0.8 μM each PCR primer–0.05 U of *Pfu* DNA polymerase/μl by using 35 cycles for amplification of gelatinase cDNAs and 25 cycles for amplification of GAPDH cDNA. The cycle conditions were 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 30 s of extension at 72°C. For Southern analysis, one-fifth of the PCR reaction product was separated on a 2.1% agarose gel, transferred to a nylon membrane (Hybond N+), and hybridized with cDNA inserts labeled with $^{32}$P by random priming. cDNAs encoding murine gelatinase A or B (33, 34) were a gift from Z. Werb, University of California, San Francisco, and the GAPDH cDNA was obtained from Clontech (Palo Alto, Calif.). Hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 50% formamide at 42°C overnight. The membranes were washed at a final stringency of 0.2× SSC and 0.1% sodium dodecyl sulfate at 68°C and were exposed to XAR-5 film for autoradiography.

**Zymography.** Proliferating cells ($10^6$ in 100-mm-diameter dishes) were shifted to serum-free medium for 2 to 3 days, after which they were given 10 ml of fresh serum-free medium. Forty-eight hours later, the conditioned medium was collected and concentrated 10- to 15-fold by using 10-kDa-cutoff filters (Millipore, Bedford, Mass.). The concentrated medium was analyzed on casein and gelatin substrate gels, as described by Fisher and Werb (10) and Talhouk et al. (41). Briefly, gels consisted of 8 to 10% polyacrylamide and 3 mg of α-casein or gelatin (Sigma)/ml. Concentrated conditioned medium was mixed with nonreducing Laemmli sample buffer and incubated at 37°C for 15 min. After electrophoresis, the gels were incubated for 1 h in 2.5% Triton X-100 at room temperature, followed by 24 to 48 h in substrate buffer (100 mM Tris-HCl [pH 7.4]–15 mM $CaCl_2$) in the absence or presence of GM6001 (0.2 mM in DMSO; supplied by Glycomed Corporation and obtained from Z. Werb [12]), EDTA (10 mM), *ortho*-phenanthroline (1 mM in DMSO; Sigma), PMSF (5 mM), or AEBSF (0.5 mM; Calbiochem). Where appropriate, control gels were incubated with buffer containing solvent only. The gels were stained with Coomassie blue for 30 min and were destained with 30% methanol–10% acetic acid. Caseinase and gelatinase activities were visible as clear bands, indicative of proteolysis of the substrate protein.

## RESULTS

**Id-1 induces an invasive, migratory phenotype in mammary epithelial cells.** SCp2 mammary epithelial cells grow as a monolayer in 5% serum. When given lactogenic hormones and basement membrane ECM in serum-free medium, they arrest growth, form three-dimensional alveolar structures, and express the milk protein β-casein (8). Alveoli formed by SCp2 cells are stable, maintaining their structure and β-casein expression for more than 2 weeks. Under these conditions, Id-1 is not expressed. By contrast, SCp2 cells that constitutively express Id-1 form poorly compacted alveoli that become increasingly disorganized; after 6 to 8 days, cells at the periphery detach from the structure and synthesize DNA (9).

Using a pooled population of SCp2 cells that constitutively express a murine Id-1 transgene (SCp2–Id-1 cells) (9), we more precisely monitored the fate of cells that detached from the alveolar structure. Within 10 days, approximately 30 to 40% of the SCp2–Id-1 alveolar structures showed substantial disintegration. Following detachment from the alveolar structure, SCp2–Id-1 cells actively invaded and migrated through the surrounding ECM (Fig. 1). The migrating cells had an elongated nuclear morphology, compared to the rounded nuclei of cells in the early stages of disaggregation. Initial detachment and invasion occurred in the absence of cell proliferation (Fig. 1A). However, 2 to 4 days after initial detachment, SCp2–Id-1 cells that had migrated extensively through the ECM were abundant, and many of these cells synthesized DNA (Fig. 1B to D). For the most part, DNA synthesis was evident only in cells that had migrated some distance from the alveolar structure. Thus, the initiation of invasion and migration was not due to resumption of growth; rather, cells resumed proliferation only after they had detached and migrated from the three-dimensional structure. As previously described (9), spheres comprised of control cells transfected with the vector alone were very stable, remaining viable and morphologically unchanged even after more than 10 days on basement membrane ECM.

To quantify the invasion and migration of SCp2–Id-1 cells, they and control cells were assayed in Boyden chambers (2). Cells were added to the upper portion of the chamber; conditioned medium from mouse fibroblasts, used as a source of chemoattractants (2), was added to the lower compartment. The porous filter separating the two compartments was coated with basement membrane ECM. After a 16- to 20-h incubation, cells that had migrated through the ECM to the lower surface of the filter were fixed, stained, and counted (Fig. 2). The 16- to 20-h incubation time ensured that only a small fraction of invasive cells migrated through the filter, which in turn ensured that the fraction of migratory cells was small enough to score reliably.

Four types of cells were compared in this assay: (i) parental SCp2 cells, (ii) SCp2 cells transfected with an insertless vector, (iii) SCp2–Id-1 cells, and (iv) SCp2 cells transfected with the Id-1 cDNA in the antisense orientation. Of these cell types, only SCp2–Id-1 cells were invasive. Under these assay conditions, none of the control (parental or insertless-vector) cells and none of the cells expressing antisense Id-1 migrated through the filter. By contrast, 0.7 to 1% of a population of one of the most invasive breast cancer cell lines (MDA-MB-231, previously described [43]) migrated through the filter, although only about 0.05% of the SCg6-transformed cells, which were previously shown to be invasive (28), migrated through the ECM to the lower surface of the filter (data not shown). In the case of the SCp2–Id-1 cells, 0.2 to 0.3% migrated through the filter. Thus, SCp2–Id-1 cells, which were transfected with a single gene, were 20 to 30% as invasive as one of the most

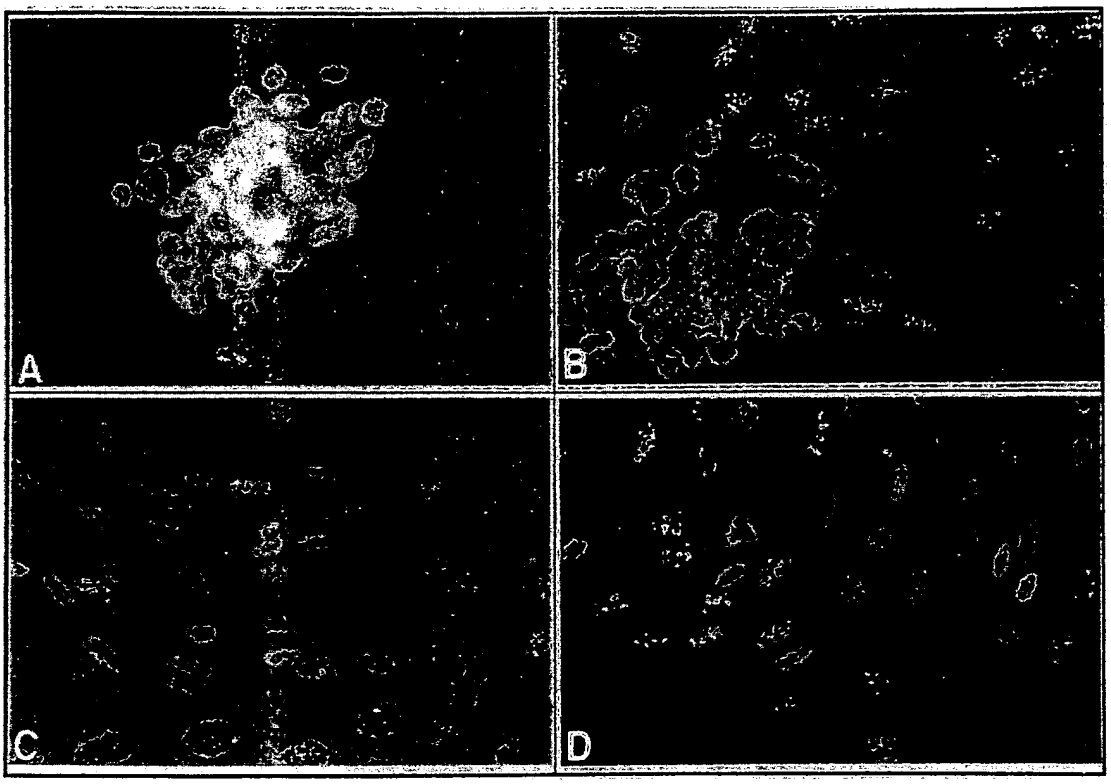


FIG. 1. Instability of the three-dimensional organization and loss of growth arrest of SCp2–Id-1 cells. A pooled population of SCp2–Id-1 cells was induced to differentiate for 8 (A), 10 (B), or 12 (C and D) days, [$^3$H]thymidine was added for 24 h preceding fixation, and the cells were then stained with DAPI and processed for autoradiography as described in Materials and Methods. Shown are the DAPI fluorescence and autoradiography. Depending on the batch of EHS ECM or Matrigel, disaggregation of the three-dimensional structures and resumption of DNA synthesis occurred 1 to 2 days earlier or later than in the experiments for which results are shown here. Magnification, ×300.

aggressive breast cancer cell lines (which harbors multiple mutations) and four- to sixfold more invasive than their SCg6-transformed counterparts.

We conclude that constitutive expression of the Id-1 gene can induce an invasive and migratory phenotype in nontransformed and nontumorigenic SCp2 mammary epithelial cells.

**Constitutive Id-1 expression is not sufficient for anchorage-independent growth or tumorigenicity.** In many model systems of malignant transformation, unregulated expression of normal or activated proto-oncogenes drives cell proliferation, and invasiveness often develops subsequent to, or concomitant with, tumorigenicity. Although Id-1 did not appear in this regard to act like a typical oncogene, we nonetheless asked whether constitutive Id-1 expression transformed SCp2 cells, using the criteria of anchorage-independent growth and tumorigenicity in nude mice.

We first tested the ability of the cells to grow in an anchorage-independent manner. As expected, control cells and cells transfected with Id-1 in the antisense orientation failed to grow in soft agar (Fig. 3A and C). Similarly, SCp2–Id-1 cells failed to form colonies in soft agar, remaining as single cells for at least 14 days (Fig. 3B). It is interesting that, in soft agar, SCp2–Id-1 cells appeared twice as large as control cells; the reason for this size increase is not known. Malignant TCL1 cells (isolated from a murine mammary tumor [28]), used as a positive control, formed large colonies after 14 days in soft agar (Fig. 3D). We conclude that constitutive expression of Id-1 does not

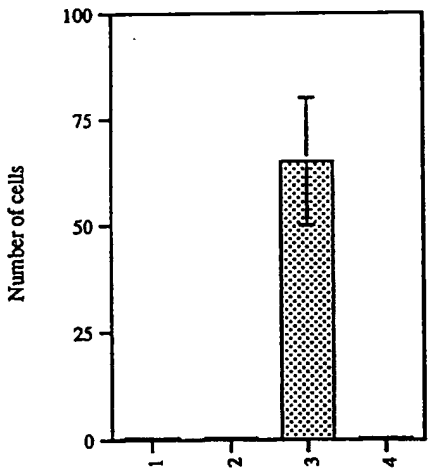


FIG. 2. SCp2–Id-1 cells invade the ECM and migrate in a Boyden chamber. Parental SCp2 cells (lane 1), SCp2 cells transfected with an insertless vector (lane 2), SCp2–Id-1 cells (lane 3), and SCp2 cells transfected with Id-1 in the antisense orientation (lane 4) were plated on ECM-coated filters in Boyden chambers; the number of cells that migrated through the filter after 16 to 20 h was determined as described in Materials and Methods. Error bars indicate standard deviations from three or four independent fields. The data shown are from one of five independent experiments which showed very similar differences among the cell types.

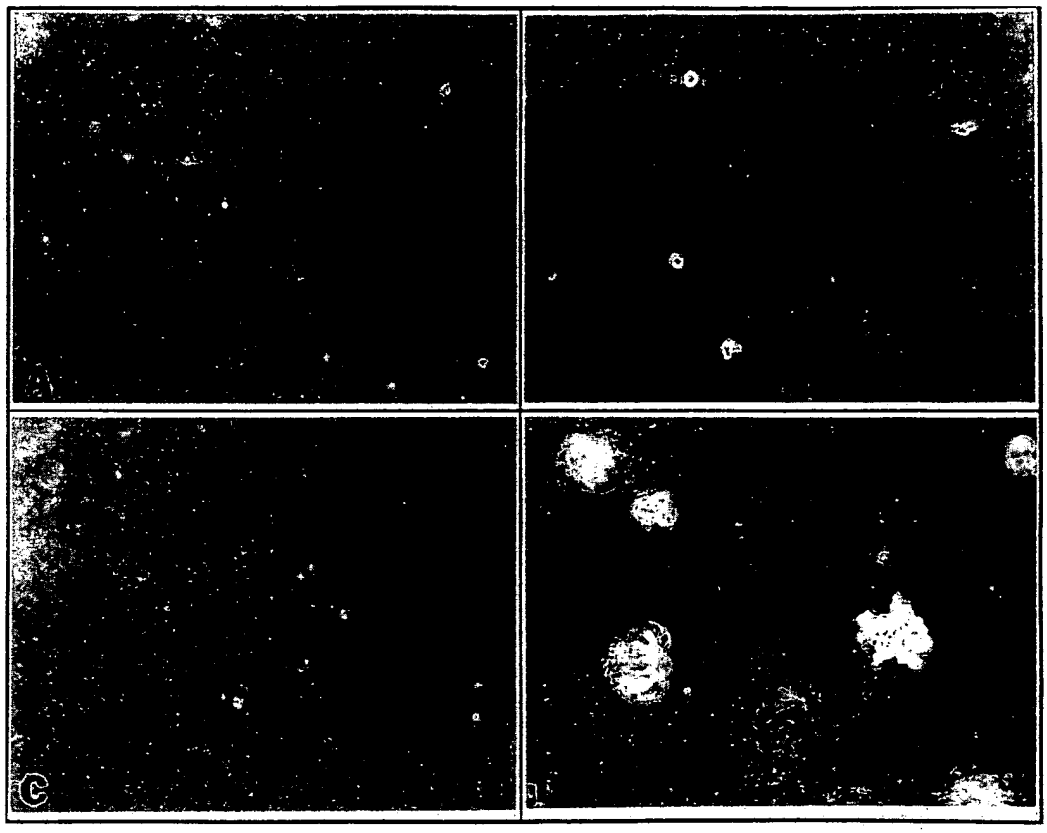

FIG. 3. SCp2–Id-1 cells do not grow in an anchorage-independent manner. Parental SCp2 cells (A), SCp2–Id-1 cells (B), SCp2 cells expressing an Id-1 antisense vector (C), and TCL1 mammary tumor cells (D) were seeded in soft agar as described in Materials and Methods and photographed 14 days later. Magnification, ×50.

induce anchorage-independent growth in SCp2 mammary epithelial cells.

We next tested SCp2–Id-1 cells for their ability to form tumors. Cells were injected subcutaneously into nude mice. The positive control, TCL1 cells, formed tumors (at least 1 $cm^3$) within 3 weeks (Table 1). The same was true for SCg6, a cell line with mesenchymal and transformed properties that was isolated from the same population from which SCp2 cells were isolated (8) (Table 1). By contrast, neither parental SCp2 cells, SCp2 cells expressing the Id-1 antisense cDNA, nor SCp2–Id-1 cells formed tumors after 5 months (Table 1).

We conclude that constitutive Id-1 expression in SCp2 mammary epithelial cells is not sufficient to lead to the transformed phenotypes of anchorage-independent growth in culture and in vivo, despite its ability to induce an invasive phenotype.

**Isolation and characterization of cloned SCp2–Id-1 cells.** The experiments described thus far used a pooled population of SCp2–Id-1 cells, which is heterogeneous with respect to Id-1 expression. To eliminate this heterogeneity and better define the role of Id-1 in inducing an invasive phenotype, we isolated single-cell-derived SCp2–Id-1 clones that expressed the Id-1 transgene to varying levels. The clones were assessed for cytokeratin filaments (a general characteristic of epithelial cells), morphology in monolayer culture, and ability to form alveolar structures in response to basement membrane ECM. In addition, RNA was isolated 5 days after the cells were exposed to basement membrane and hormones and was analyzed for Id-1 and β-casein mRNA. The Id-1 transgene mRNA was distinguishable from the endogenous Id-1 mRNA by its slightly larger size; the endogenous transcript was barely detectable under these conditions (9).

One subclone, SCp2–Id-1A cells, did not express detectable Id-1 transgene mRNA (Fig. 4a, lane 1). These cells grew as compact colonies in monolayer culture and expressed cytokeratin filaments (Fig. 4b, panel B). They also differentiated similarly to untransfected SCp2 cells, as judged by their ability to express high levels of β-casein mRNA (Fig. 4a, lane 1) and form stable alveolar structures (data not shown), like untransfected SCp2 cells. These cells were therefore used as negative controls.

SCp2–Id-1B and SCp2–Id-1C cells expressed moderate levels of the Id-1 transgene, which were below the levels of Id-1

TABLE 1. SCp2–Id-1 cells are not tumorigenic[a]

| Cell type | No. of tumors/no. of sites injected (time) |
|---|---|
| TCL1 | 4/4 (after 3 wk) |
| SCg6 | 4/4 (after 3 wk) |
| SCp2 | 0/4 (after 5 mo) |
| SCp2–antisense Id-1 | 0/4 (after 5 mo) |
| SCp2–Id-1 | 0/4 (after 5 mo) |

[a] Cells ($4 \times 10^6$ per site) were injected into two sites per animal, and two animals were used for each cell type, as described in Materials and Methods. The tumors that developed were at least 1 $cm^3$.

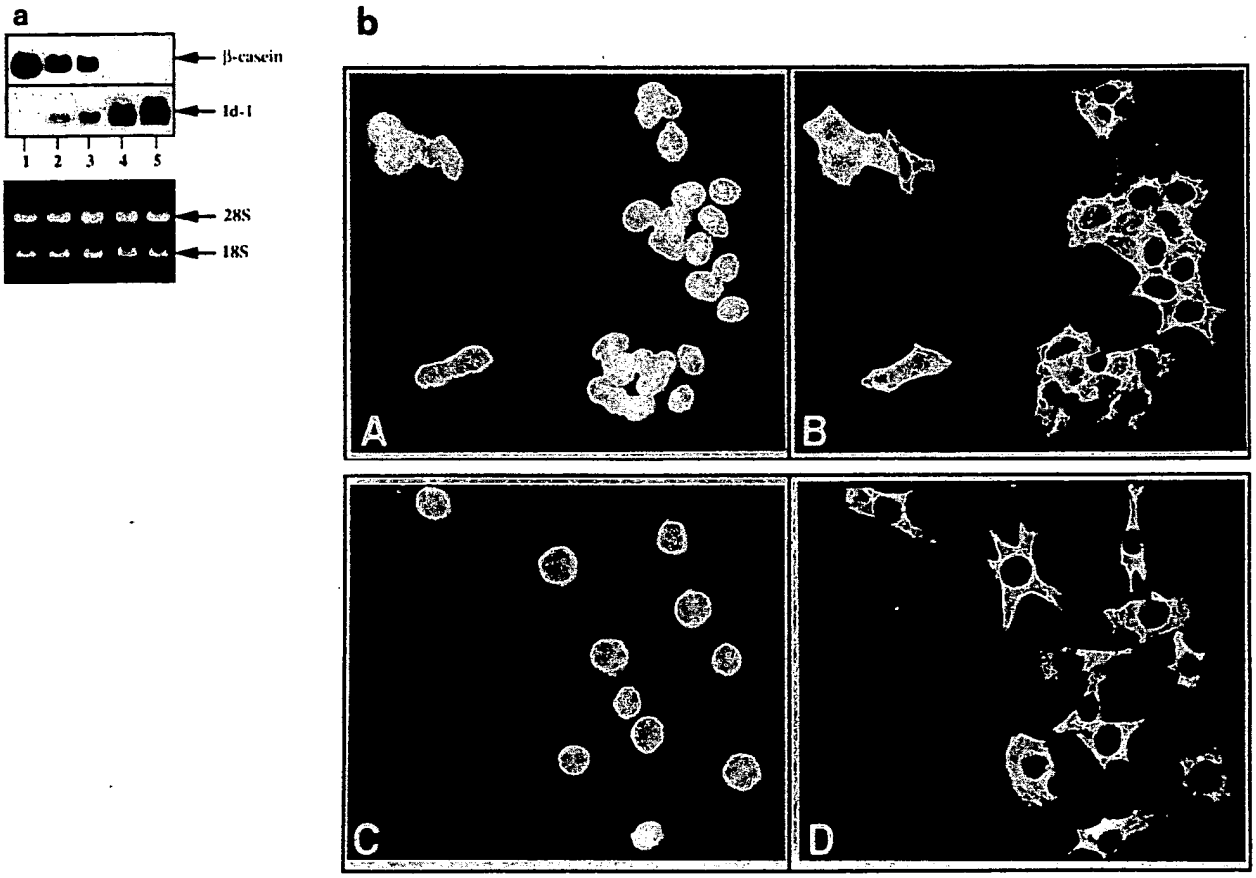


FIG. 4. Characterization of SCp2 cell clones expressing constitutive Id-1. (a) SCp2–Id-1 cells were plated at limiting dilution, and five independent clones (SCp2–Id-1A through SCp2–Id-1E) were isolated and amplified. Cells from each of these clones were exposed to basement membrane and hormones for 5 days and were analyzed for expression of the Id-1 transgene and β-casein mRNA, as described in Materials and Methods. Shown are the autoradiogram of the Northern blot and the ethidium bromide-stained Northern gel made to confirm RNA integrity and quantitation. Lane 1, SCp2–Id-1A; lane 2, SCp2–Id-1B; lane 3, SCp2–Id-1C; lane 4, SCp2–Id-1D; lane 5, SCp2–Id-1E. (b) SCp2–Id-1A (A and B) and SCp2–Id-1E (C and D) cells were grown in monolayer culture, fixed, and stained with DAPI (A and C) or processed for immunofluorescence by using a pan-keratin antibody (B and D), as described in Materials and Methods.

mRNA expressed by proliferating control cells. These cells expressed lower levels of β-casein mRNA than SCp2–Id-1A cells (Fig. 4a, lanes 2 and 3), but they expressed cytokeratin filaments and formed alveolar structures (data not shown).

Finally, SCp2–Id-1D and SCp2–Id-1E cells expressed high levels of the Id-1 transgene and undetectable levels of β-casein (Fig. 4a, lanes 4 and 5). In monolayer culture, SCp2–Id-1E cells were less cuboidal and grew as more-dispersed entities than SCp2–Id-1A cells (Fig. 4b, panels C and D). Their failure to express β-casein was not due to a loss of epithelial characteristics. SCp2–Id-1E cells, which expressed the highest levels of Id-1, as well as SCp2–Id-1D cells (data not shown), expressed cytokeratin filaments (Fig. 4b, panel D). However, SCp2–Id-1D and SCp2–Id-1E cells, like the pooled SCp2–Id-1 cells, formed only loose alveolar structures, from which they eventually detached and invaded the ECM (see Fig. 7) (data not shown).

These results confirm in cloned populations that mammary epithelial cells constitutively expressing Id-1 do not undergo a complete epithelial-to-mesenchymal transition; they retain some epithelial-cell characteristics (such as keratin expression) but fail to functionally differentiate and to maintain three-dimensional organization on the ECM. SCp2–Id-1A and SCp2–Id-1E cells, which express undetectable and high levels of the Id-1 transgene, respectively, were used for further studies.

**A potentially novel metalloproteinase is secreted by Id-1-expressing cells.** The ability of SCp2–Id-1 cells to invade the ECM suggested that Id-1 might induce expression of ECM-degrading proteases. The major classes of proteases that degrade ECM are serine, cysteine, and aspartyl proteases, and metalloproteinases (10). The $Zn^{2+}$-containing, $Ca^{2+}$-stabilized MMPs are of particular interest because they are implicated in the remodeling of the mammary gland during involution (29, 41) and the initial steps of tumor-cell invasion (25). Of the major MMPs, interstitial collagenase (56 kDa) and gelatinases A (72 kDa) and B (92 kDa) are detectable on gelatin substrate gels, whereas stromelysins (57 kDa for stromelysin-1) and matrilysin (30 kDa) are detectable on casein substrate gels (reviewed by Fisher and Werb [10]; see also reference 22).

We examined the secretion of proteases by SCp2–Id-1A and SCp2–Id-1E cells, using conditioned medium and gelatin or casein substrate gel zymography. Cells were incubated in serum-free medium for 3 days prior to collection of conditioned medium for zymography. Under these conditions, the endogenous Id-1 gene is not expressed (9), and SCp2–Id-1A and

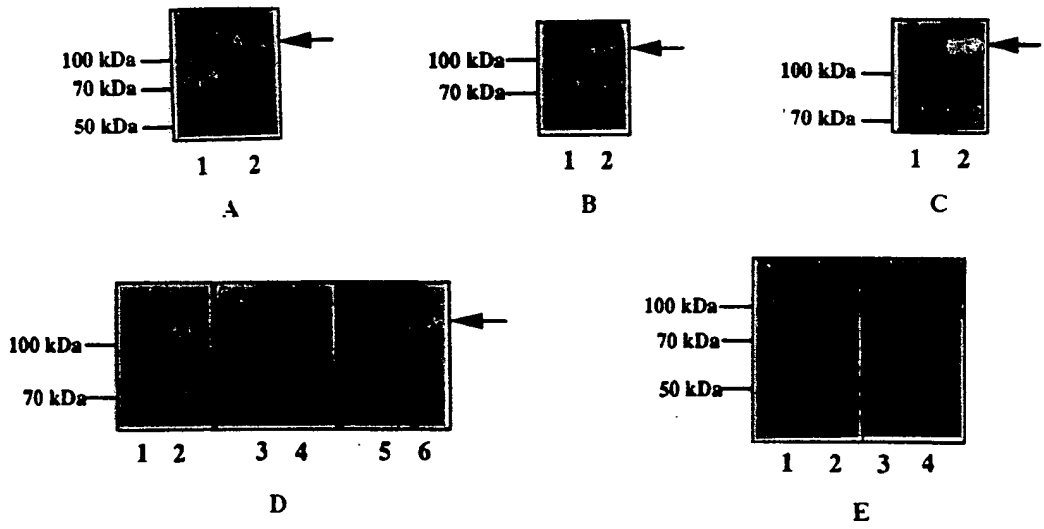


FIG. 5. Expression of a 120-kDa gelatinase by Id-1-expressing mammary epithelial cells. (A) Gelatin zymogram of conditioned media from SCp2–Id-1A (lane 1) and SCp2–Id-1E (lane 2) cells. Cells were cultured in serum-free medium, and conditioned media were harvested and analyzed on a gelatin substrate gel, as described in Materials and Methods. (B) Gelatin zymogram of conditioned media from SCp2–Id-1A cells either growth arrested by serum deprivation (lane 1) or growing in 5% serum (lane 2). (C) Gelatin zymogram of conditioned media from control SCp2 cells (lane 1) and an uncloned SCp2–Id-1-transfected pooled population (lane 2). Cells were cultured in serum-free medium. (D) Gelatin zymogram of SCp2–Id-1A (lanes 1, 3, and 5) and SCp2–Id-1E (lanes 2, 4, and 6) cell-conditioned media incubated with DMSO (lanes 1 and 2), the MMP inhibitor GM6001 (0.2 mM in DMSO) (lanes 3 and 4), or the serine proteinase inhibitor PMSF (5 mM in DMSO) (lanes 5 and 6). (E) Casein zymogram of conditioned media from SCp2–Id-1A (lanes 1 and 3) and SCp2–Id-1E (lanes 2 and 4) cells incubated with DMSO (lanes 1 and 2) or GM6001 (lanes 3 and 4). In panels A through D, arrows mark the positions of the 120-kDa MMP.

SCp2–Id-1E express undetectable and high levels of the Id-1 transgene, respectively.

Gelatin substrate gels showed that SCp2–Id-1A and SCp2–Id-1E cells differed only in the secretion of a high-molecular-mass (approximately 120-kDa) gelatinase. The 120-kDa gelatinase was abundantly expressed by serum-deprived SCp2–Id-1E cells (Fig. 5A, lane 2) as well as SCp2–Id-1D cells (data not shown). Secretion of this 120-kDa gelatinase was not due to clonal variation. Conditioned medium from the uncloned SCp2–Id-1 pooled population also showed a gelatinase of ~120 kDa (Fig. 5C, lane 2). This gelatinase was undetectable in serum-deprived SCp2–Id-1A (Fig. 5A, lane 1) and control SCp2 (Fig. 5C, lane 1) cells. Thus, secretion of a 120-kDa gelatinase correlated with Id-1 expression.

Secretion of the 120-kDa gelatinase correlated with expression of the endogenous Id-1 gene as well as with that of the Id-1 transgene. Thus, the 120-kDa proteinase was secreted by SCp2–Id-1A cells (in which expression of the Id-1 transgene is undetectable) while they were proliferating in monolayer culture (Fig. 5B, lane 2). Under these conditions, the endogenous Id-1 gene is expressed at high levels (9).

The 120-kDa gelatinase had characteristics of an MMP. It was sensitive to the MMP inhibitors GM6001 (Fig. 5D, lane 4), EDTA, and *ortho*-phenanthroline (data not shown). By contrast, it was insensitive to the serine protease inhibitors PMSF (Fig. 5D, lane 6) and AEBSF (data not shown). The 120-kDa MMP appeared to be the only MMP secreted by Id-1-expressing cells. The two gelatinases with apparent molecular sizes of 70 and 90 kDa, which were expressed by both SCp2–Id-1A and SCp2–Id-1E cells, were not inhibited by any of the MMP inhibitors GM6001 (Fig. 5D, lane 4), *ortho*-phenanthroline, and EDTA (data not shown), and therefore neither is likely to be gelatinase A or B.

Casein substrate gels showed one major caseinase of approximately 50 kDa that was expressed by both SCp2–Id-1A and SCp2–Id-1E cells. This protease was not inhibited by the metalloproteinase inhibitor GM6001 (Fig. 5E). Therefore, it is most likely not the metalloproteinase stromelysin-1.

To definitively rule out the possibility that gelatinases A and B were expressed in SCp2 cells, as well as the possibility that the 120-kDa MMP was a complex between gelatinase B and its carrier protein (21), we analyzed RNA by PCR and Southern blotting for gelatinase-A and -B mRNAs. SCp2–Id-1A cells, which do not express the Id-1 transgene, SCp2–Id-1E cells, which express high levels of the Id-1 transgene, and the mesenchyme-like mammary SCg6 cells were deprived of serum for 3 days before RNA was extracted and synthesized into cDNA for PCR analysis (Fig. 6). The 326-bp PCR product expected from the gelatinase-A cDNA and the 190-bp product expected for the gelatinase-B cDNA were detected only in SCg6 cells (Fig. 6, lane 3). We conclude that SCp2 cells, whether or not they express Id-1, do not express gelatinase A or B and that therefore the 120-kDa gelatinase is not a gelatinase B-containing complex (21).

We conclude that SCp2 mammary epithelial cells secrete a single detectable MMP, having an apparent molecular size of 120 kDa, when they express Id-1. This MMP does not belong to the stromelysin subclass of MMPs, which degrades casein, but rather is a type IV collagenase MMP family member and thus degrades gelatin, a denatured collagen.

**The Id-1-related MMP is essential for the invasive phenotype of SCp2 cells.** Because the 120-kDa MMP appears to be the only proteinase whose secretion correlates with Id-1 expression, and constitutive Id-1 expression renders cells invasive, we explored the possibility that this MMP is critical for the invasive phenotype of mammary epithelial cells.

We first tested the abilities of SCp2–Id-1A and SCp2–Id-1E cells to invade basement membrane ECM in a Boyden chamber invasion assay (Fig. 7). SCp2–Id-1A cells, like untransfected SCp2 cells (Fig. 2), were not invasive, or only minimally invasive, in this assay (Fig. 7). Under the assay conditions, the endogenous Id-1 gene is not expressed and SCp2–Id-1A cells

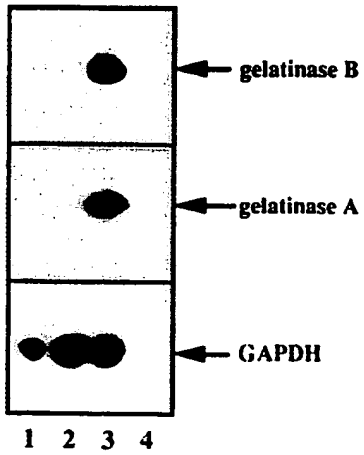


FIG. 6. SCp2 cells do not express gelatinase A or B. SCp2 cells were serum deprived for 3 days before RNA was extracted, transcribed into cDNA, and analyzed by PCR for gelatinase-A and -B cDNA sequences, as described in Materials and Methods. Arrows indicate the positions of the amplified products for gelatinases A and B and the control gene, encoding GAPDH. Lane 1, SCp2-Id-1A cells; lane 2, SCp2-Id-1E cells; lane 3, SCg6 cells; lane 4, no cDNA control.

express undetectable levels of the Id-1 transgene. By contrast, SCp2–Id-1E cells, like uncloned SCp2–Id-1 cells (Fig. 2), were demonstrably invasive under the same conditions, consistent with the high levels of the Id-1 transgene expressed by these cells.

To test the role of the 120-kDa MMP in the invasive phenotype induced by Id-1, we used MMP inhibitors in the invasion assay. We first tested the toxicities of two compounds, GM6001 and phenanthroline. SCp2 cells were treated with either compound, the solvent (DMSO), or nothing for the duration of the invasion assays (20 h), and viability was assessed by trypan blue exclusion. There were no differences in viability among untreated, DMSO-treated, and GM6001-treated cells (data not shown). By contrast, all the phenanthroline-treated cells died within 20 h of treatment. We therefore used GM6001 in the invasion assay. GM6001 reduced the invasiveness of SCp2–Id-1E cells about fourfold (Fig. 7, lane 3). Because the 120-kDa gelatinase is the only detectable MMP expressed by these cells, this result suggests that much of the invasive phenotype induced by Id-1 can be attributed to the 120-kDa MMP.

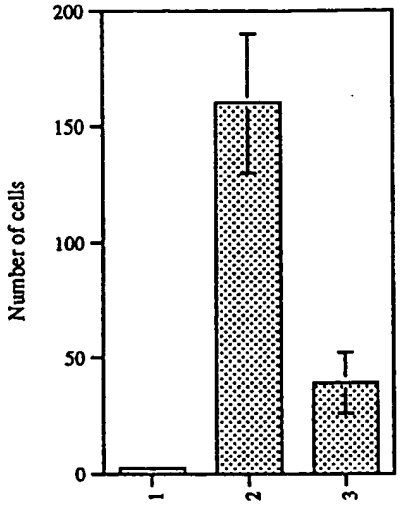


FIG. 7. The invasive phenotype of Id-1-expressing cells is repressed by an MMP inhibitor. SCp2–Id-1A cells in 0.5% DMSO (lane 1), SCp2–Id-1E cells in 0.5% DMSO (lane 2), and SCp2–Id-1E cells in GM6001 (200 μM; 0.5% DMSO) (lane 3) were plated on ECM-coated filters in Boyden chambers, and the numbers of cells that migrated through the membrane after 16 to 20 h were determined as described in Materials and Methods and the legend to Fig. 2. Error bars indicate standard deviations from three to four independent fields; the data shown are from one of three independent experiments.

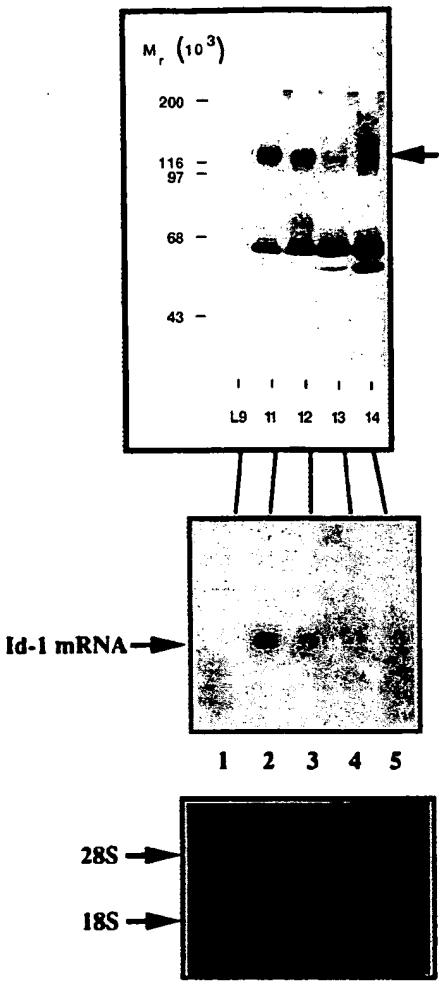


FIG. 8. Correlation between expression of the 120-kDa gelatinase and Id-1 in vivo. Cell extracts were prepared from lactating and involuting glands (as described by Talhouk et al. [41]) and analyzed by gelatin zymography. In the upper panel, the arrow marks the position of the 120-kDa gelatinase. RNA was isolated from mammary glands at the same stages and analyzed on Northern blots for Id-1 mRNA (middle panel). Lane 1, day 9 of lactation; lanes 2 through 5, days 1, 2, 3, and 4 of involution, respectively. The ethidium bromide-stained gel is shown in the lower panel to confirm RNA integrity and quantitation.

**Id-1 and the 120-kDa gelatinase are expressed during mammary gland involution.** In studying proteases during mouse mammary-gland development, Talhouk et al. (41) described a gelatinase having a molecular size greater than 110 kDa that was not expressed during lactation (Fig. 8, top panel, lane 1) but was expressed during the early stages of involution (days 1 and 2 [lanes 2 and 3], declining by day 3 [lane 4]). The identity

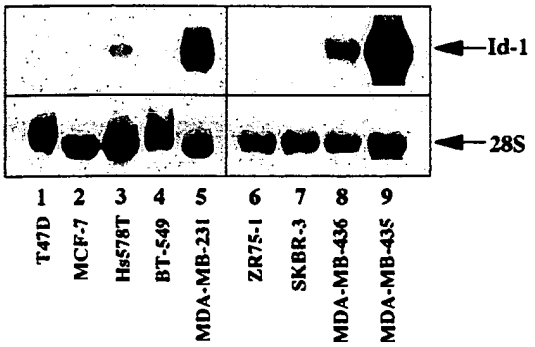


FIG. 9. Id-1 expression in nine human breast cancer cell lines. Cells were cultured in serum-free medium for 2 days before RNA was extracted and subjected to Northern blotting. The blots were then hybridized with a human Id-1 cDNA probe. Hybridization to the 28S rRNA is also indicated.

or function of this gelatinase was not determined or discussed. To explore the possibility that this gelatin-degrading proteinase may be the 120-kDa MMP expressed by Id-1-expressing cells, we isolated RNA from lactating and involuting mouse mammary glands and measured Id-1 expression by Northern analysis (Fig. 8, lower panels). Id-1 mRNA was undetectable in the lactating gland (lane 1) but was highly expressed early in involution (day 1 and 2 [lanes 2 and 3]); Id-1 expression began to decline by the 3rd day of involution (lane 4). Thus, the correlation between the expression of Id-1 and a 120-kDa gelatinase observed in mammary epithelial-cell cultures is also seen in the intact mammary gland during involution.

**Id-1 and 120-kDa gelatinase expression in invasive human breast cancer cells.** Our finding that ectopic Id-1 expression induced a 120-kDa gelatinase and an invasive phenotype in mouse mammary epithelial cells suggested that Id-1 and its associated gelatinase could, at least in some instances, contribute to human breast cancer progression. To begin to explore this possibility, we examined human breast cancer cell lines exhibiting varying degrees of invasiveness in culture and in vivo.

We examined four differentiated, essentially noninvasive breast cancer cell lines, T47D, MCF-7, ZR75-1, and SKBR-3 (43), and five poorly differentiated and invasive cell lines, Hs578T, BT-549, MDA-MB-231, MDA-MB-436, and MDA-MB-435 (27, 30, 43, 50) (Fig. 9). These cell lines have been evaluated for invasiveness in culture, by using the Boyden chamber assay (2), and in vivo, by using metastatic tumor formation in nude mice (43). By both assays (under serum-free and/or estrogen-free conditions), T47D, MCF-7, ZR75-1, and SKBR-3 cells were noninvasive. By contrast, Hs578T, BT-549, and particularly MDA-MB-231, MDA-MB-436, and MDA-MB-435 cells were highly invasive by both assays. We confirmed the reported invasive potentials of these cells, using the Boyden chamber assay (data not shown).

When cells were cultured in serum-free medium for 2 days, Id-1 mRNA was undetectable in the noninvasive T47D, MCF-7, ZR75-1, and SKBR-3 cells (Fig. 9, lanes 1, 2, 6, and 7) but was easily detectable in the highly invasive MDA-MB-231, MDA-MB-436, and MDA-MB-435 cells (Fig. 9, lanes 5, 8, and 9). Of the other invasive cells, Hs578T expressed low levels of Id-1 mRNA (Fig. 9, lane 3), whereas Id-1 mRNA was undetectable in BT-549 (Fig. 9, lane 4). Thus, the invasive potential of the human breast cancer cell lines MDA-MB-231, MDA-MB-436, MDA-MB-435, and, to a lesser extent, Hs578T could, at least in part, derive from unregulated expression of Id-1 and its associated 120-kDa gelatinase.

Consistent with this idea, a 120-kDa gelatinase was detected in conditioned media from the invasive cells that expressed Id-1 (Hs578T, MDA-MB-231, MDA-MB-436, and MDA-MB-435; Fig. 10, lanes 3, 5, 8, and 9, respectively). This gelatinase was not detected in conditioned media from the noninvasive cell lines T47D, MCF-7, ZR75-1, and SKBR-3 (Fig. 10, lanes 1, 2, 6, and 7) or from the invasive cell line that did not express Id-1 (BT-549; Fig. 10, lane 4). The 120-kDa gelatinase expressed by the human breast cancer cells comigrated with the 120-kDa gelatinase expressed by Id-1-transfected SCp2 cells (Fig. 10, lane C). As previously reported (1), the 72- and/or 92-kDa gelatinases were detected in most of these human cell lines, whether or not they were invasive. Despite the secretion of these gelatinases by the cells, only the 120-kDa gelatinase-expressing cells were invasive in the Boyden chamber invasion assay (reference 43 and data not shown). The exception was the invasive BT-549 cell line, which neither expressed Id-1 mRNA nor secreted the 120-kDa gelatinase. BT-549 cells express many MMPs (by zymography), including high levels of membrane type 1 MMPs (11).

Thus, among nine human breast tumor cells examined, only Id-1-expressing cells also expressed the 120-kDa gelatinase, and all Id-1-negative cells failed to express the 120-kDa gelatinase. Moreover, the Id-1- and 120-kDa gelatinase-expressing cells were all invasive in culture and in vivo.

## DISCUSSION

The mammary gland is one of the few organs that undergo striking morphological and functional changes during adult life, particularly during pregnancy, lactation, and involution. In both humans and mice, fetal, virgin adult, and pregnant mammary glands undergo extensive temporal and spatial remodeling, which entails invasion, migration, and relocation of cells to generate the ductal and alveolar structures of the gland. Once lactation is terminated, there is additional and extensive tissue remodeling as the gland returns to its resting state. In recent years, progress has been made in elucidating the mechanisms that regulate mammary gland-specific gene expression and the transformation of mammary epithelial cells to malignancy (3, 39). However, much less is known about the mechanisms, particularly the transcriptional mechanisms, that regulate the development and remodeling of the normal mammary gland.

**SCp2 cells as a model for normal mammary epithelial cells.** SCp2 is an immortal murine cell line that nonetheless expresses many characteristics of epithelial cells in the pregnant and lactating mammary gland. SCp2 cells proliferate in mono-

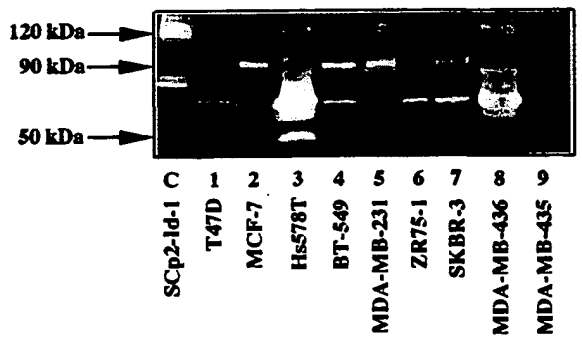


FIG. 10. Expression of a 120-kDa gelatinase in Id-1-positive cells. Serum-free conditioned media from SCp2–Id-1 transfected cells (lane C [control]) and nine human breast cancer cell lines (lanes 1 to 9) were analyzed by gelatin zymography.

layer culture in response to serum growth factors but arrest growth, form alveolar structures, and express milk proteins in response to lactogenic hormones and basement membrane components. Arrested growth is necessary, but not sufficient, for differentiation. The differentiation of SCp2 cells in culture is remarkably similar to the differentiation of mammary epithelial cells in vivo (8). Here, we extend this similarity to expression of a 120-kDa MMP that appears to be controlled by Id-1, a negative regulator of bHLH transcription factors (4).

**Id-1 as a negative regulator of mammary epithelial-cell differentiation.** During proliferation, but not during arrested growth or differentiation, SCp2 cells express Id-1. The expression of Id-1 and that of the milk protein β-casein are inversely correlated in cultured SCp2 cells (9), as well as in the mammary gland in virgin, pregnant, and lactating mice (9a). Indeed, Id-1 is a negative regulator of the functional differentiation of SCp2 cells. When constitutively expressed, Id-1 prevents the strong cell-cell contacts typical of differentiated cells and blocks milk protein expression. Although the precise mechanism by which Id-1 inhibits differentiation is not known, it is clear that it does not act by preventing the growth arrest induced by hormones and ECM (9).

Id-1 is presumed to repress differentiation by inhibiting one or more bHLH transcription factors. By analogy with the role of bHLH proteins in the differentiation of muscle, neuronal, and lymphoid cells (24, 40, 47), bHLH transcription factors may be required for differentiation-specific gene expression in the mammary gland. However, our results suggest an additional role for bHLH proteins in the mammary gland: repression of a 120-kDa MMP, whose activity permits the epithelial cells to migrate and invade the ECM.

**An Id-1-regulated gelatinase expressed by mammary epithelial cells.** Id-1 expression, whether originating from the endogenous gene or a transgene, correlated strongly with the expression of a 120-kDa gelatinase having the characteristics of an MMP. This protease appeared to be the only metalloproteinase expressed by SCp2 mammary epithelial cells. The well-characterized MMPs stromelysin and gelatinases A and B (72- and 92-kDa type IV collagenases) were not expressed by SCp2 cells. By contrast, gelatinases A and B were expressed by SCg6, a stroma-like cell line derived from the same culture from which SCp2 cells were cloned (8). SCg6 cells also express stromelysin-1 (28). These findings suggest that the expression of stromelysin and gelatinases A and B during involution of the mammary gland may derive from the nonepithelial cells in the tissue (29).

The epithelial cells of the mammary gland, on the other hand, may express the 120-kDa MMP. Talhouk et al. (41) described a gelatinase with an apparent molecular size exceeding 110 kDa that was expressed during the early stages of involution. We found that Id-1 mRNA was not expressed during lactation, when the 120-kDa gelatinase is undetectable, but was expressed early in involution (days 1 and 2). We suggest that this gelatinase may be the 120-kDa MMP identified in Id-1-expressing SCp2 cells. Thus, there is a correlation between Id-1 expression and secretion of a 120-kDa gelatinase in vivo, as well as in cultured cells.

**The Id-1-regulated gelatinase is critical for epithelial-cell invasiveness.** SCp2 cells arrest growth when in contact with basement membrane ECM. Under these conditions, Id-1 is not expressed, the cells maintain strong contacts, and they do not invade the surrounding ECM (9). Constitutive Id-1 expression did not prevent the growth arrest but conferred an invasive phenotype on the cells. Only after Id-1-expressing SCp2 cells had invaded the ECM did they resume proliferation. Thus, Id-1 appeared to be a regulator of the invasive phenotype rather than a stimulator of cell proliferation per se. This invasive phenotype, in turn, appeared to depend primarily on the 120-kDa gelatinase (MMP). This MMP was the only detectable target of GM6001, a nontoxic MMP inhibitor (12), and GM6001 effectively inhibited the invasive phenotype of Id-1-expressing cells. Thus, Id-1 and its related 120-kDa MMP were key regulators of the invasive phenotype of SCp2 cells. During involution, the Id-1-associated MMP may participate in remodeling the gland in vivo. We suggest that Id-1 and its related MMP may be key regulators of the transient invasive phenotype acquired by the epithelial cells during certain stages of normal mammary-gland development and remodeling.

**Id-1 and the 120-kDa gelatinase in tumor cell invasion.** The invasive phenotype induced by Id-1 was not the result of malignant transformation. Id-1-expressing SCp2 cells did not grow in an anchorage-independent manner and did not form detectable tumors in nude mice. Thus, Id-1 differs from oncogenes such as v-Ha-ras, which converts mouse mammary epithelial cells into invasive but also tumorigenic cells (13). Furthermore, Id-1 did not induce an invasive phenotype by converting cells to a stromal or mesenchymal phenotype. Id-1-expressing SCp2 cells maintained their epithelial characteristics, such as keratin expression, and did not express stromal MMPs. Thus, the action of Id-1 differs from that of genes of the ets family. c-Ets, a transcription factor expressed by stromal fibroblasts, promotes epithelial tumor cell invasion (48) by inducing stromal MMPs such as stromelysin-1. E1AF, a new member of the ets family, induces an invasive and migratory phenotype in human MCF-7 breast cancer cells (19), presumably by inducing gelatinase B as well as stromelysin-1.

Although the Id-1-induced invasive phenotype was not a consequence of malignant transformation, our results with human breast cancer cells suggest that constitutive Id-1 expression, and its associated 120-kDa gelatinase, may play a role in the invasive phenotype of at least some aggressive human breast tumors. We hypothesize that Id-1 and the 120-kDa gelatinase may constitute a thus far unrecognized pathway for tumor cell invasion. A very recent report (32) suggests that the Id-1–120-kDa gelatinase pathway we describe here may be of substantial clinical importance. In that report, a gelatinase of approximately the same size as the one described here was detected in urine from metastatic breast cancer patients but not in urine specimens from patients with other types of cancer. The authors acknowledge that the identity of this gelatinase is as yet unknown but suggest that it might serve as a predictor of metastatic breast cancer. By contrast, the 72- and 92-kDa gelatinases detected in urine were suggested to serve as predictors of organ-confined cancers. These suggestions are consistent with our results showing that the 72- and/or 92-kDa gelatinase is expressed by differentiated and noninvasive human breast cancer cells, whereas the 120-kDa gelatinase is expressed only in invasive breast cancer cells.

In conclusion, we propose that Id-1 regulates the invasive phenotype of breast epithelial cells, in part through the activity of a 120-kDa gelatinase, during normal mammary-gland development and remodeling. Although this phenotype is not necessarily linked to tumorigenesis, it may well be reactivated during progression toward malignancy in some breast cancers, for example, during the transition from an in situ to an invasive carcinoma. We do not yet know whether Id-1 induces the 120-kDa gelatinase by directly inactivating a bHLH repressor of the gene or whether it acts indirectly by altering the expression of other genes. We are currently attempting to clone the 120-kDa gelatinase in order to answer these questions.

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P.-Y.D. and C.Q.L. contributed equally to this work.

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## REFERENCES

1. **Abbas Abidi, S. M., E. W. Howard, J. J. Dmytryk, and J. T. Pento.** 1997. Differential influence of antiestrogens on the in vitro release of gelatinases (type IV collagenases) by invasive and non-invasive breast cancer cells. Clin. Exp. Metastasis **15**:432–439.
2. **Albini, A., Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aaronson, J. M. Kozlowski, and R. N. McEwan.** 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. **47**:3239–3245.
3. **Band, V.** 1995. Preneoplastic transformation of human mammary epithelial cells. Semin. Cancer Biol. **6**:185–192.
4. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
6. **Cross, J. C., M. L. Flannery, M. A. Blanar, E. Steingrimsson, N. A. Jenkins, N. G. Copeland, W. J. Rutter, and Z. Werb.** 1995. Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. Development **121**:2513–2523.
7. **Danielson, K. G., C. J. Oborn, E. M. Durban, J. S. Buetel, and D. Medina.** 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc. Natl. Acad. Sci. USA **81**: 3756–3760.
8. **Desprez, P. Y., C. Roskelley, J. Campisi, and M. J. Bissell.** 1993. Isolation of functional cell lines from a mouse mammary epithelial cell strain: the importance of basement membrane and cell-cell interaction. Mol. Cell. Differ. **1**:99–110.
9. **Desprez, P. Y., E. Hara, M. J. Bissell, and J. Campisi.** 1995. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. Mol. Cell. Biol. **15**:3398–3404.

9a. **Desprez, P. Y., C. Sympson, J. Campisi, and M. J. Bissell.** Unpublished data.

10. **Fisher, S. J., and Z. Werb.** 1995. The catabolism of extracellular matrix components, p. 261–287. *In* M. A. Haralson and J. R. Hassell (ed.), Extracellular matrix: a practical approach. IRL Press Ltd., Oxford, United Kingdom.
11. **Gilles, C., M. Polette, M. Seiki, P. Birembaut, and E. W. Thompson.** 1997. Implication of collagen type I-induced membrane type 1-matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. Lab. Investig. **76**:651–660.
12. **Grobelny, D., L. Poncz, and R. E. Galardy.** 1992. Inhibition of human skin fibroblast collagenase, thermolysin, and Pseudomonas aeruginosa elastase by peptide hydroxamic acids. Biochemistry **31**:7152–7154.
13. **Gunzburg, W. H., B. Salmons, A. Schlaeffli, S. Moritz-Legrand, W. Jones, N. H. Sarkar, and R. Ullrich.** 1988. Expression of the oncogenes mil and ras abolishes the in vivo differentiation of mammary epithelial cells. Carcinogenesis **9**:1849–1856.
14. **Hara, E., T. Yamaguchi, H. Nojima, T. Ide, J. Campisi, H. Okayama, and K. Oda.** 1994. Id-related genes encoding HLH proteins are required for G1 progression and are repressed in senescent human fibroblasts. J. Biol. Chem. **269**:2139–2145.
15. **Hara, E., J. A. Uzman, G. P. Dimri, J. O. Nehlin, A. Testori, and J. Campisi.** 1996. The helix-loop-helix Id-1 and a retinoblastoma protein binding mutant of SV40 T antigen synergize to reactivate DNA synthesis in senescent human fibroblasts. Dev. Genet. **18**:161–172.
16. **Haslam, S. Z.** 1989. The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. Endocrinology **125**:2766–2772.
17. **Iavarone, A., P. Garg, A. Lasorella, J. Hsu, and M. A. Israel.** 1994. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. **8**:1270–1284.
18. **Jen, Y., H. Weintraub, and R. Benezra.** 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes Dev. **6**:1466–1479.
19. **Kaya, M., K. Yoshida, F. Higashino, T. Mitaka, S. Ishii, and K. Fujinaga.** 1996. A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene **12**:221–227.
20. **Kingston, R. E.** 1989. Transcription control and differentiation: the HLH family, c-myc and C/EBP. Curr. Opin. Cell Biol. **1**:1081–1087.
21. **Kjeldsen, L., A. H. Johnsen, H. Sengelov, and N. Borregaard.** 1993. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J. Biol. Chem. **268**:10425–10432.
22. **Kleiner, D. E., and W. G. Stetler-Stevenson.** 1994. Quantitative zymography: detection of picogram quantities of gelatinases. Anal. Biochem. **218**:325–329.
23. **Kreider, B. L., R. Benezra, G. Rovera, and T. Kadesch.** 1992. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science **255**:1700–1702.
24. **Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Weintraub.** 1995. Conversion of Xenopus ectoderm into neurons by neuroD, a basic helix-loop-helix protein. Science **268**:836–844.
25. **Liotta, L. A., P. S. Steeg, and W. G. Stetler-Stevenson.** 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell **64**:327–336.
26. **Lister, J., W. C. Forrester, and M. H. Baron.** 1995. Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. J. Biol. Chem. **270**: 17939–17946.
27. **Liu, Z., M. G. Brattain, and H. Appert.** 1997. Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. Biochem. Biophys. Res. Commun. **231**:283–289.
28. **Lochter, A., A. Srebrow, C. J. Sympson, N. Terracio, Z. Werb, and M. J. Bissell.** 1997. Misregulation of stromelysin-1 expression in mouse mammary tumor cells accompanies acquisition of stromelysin-1-dependent invasive properties. J. Biol. Chem. **272**:5007–5015.
29. **Lund, L. R., J. Romer, N. Thomasset, H. Solberg, C. Pyke, M. J. Bissell, K. Dano, and Z. Werb.** 1996. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development **122**:181–193.
30. **Maemura, M., S. K. Akiyama, V. L. Woods, Jr., and R. B. Dickson.** 1995. Expression and ligand binding of alpha 2 beta 1 integrin on breast carcinoma cells. Clin. Exp. Metastasis **13**:223–235.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Moses, M. A., D. Wiederschain, K. R. Loughlin, D. Zurakowski, C. C. Lamb, and M. R. Freeman.** 1998. Increased incidence of matrix metalloproteinases in urine of cancer patients. Cancer Res. **58**:1395–1399.
33. **Reponen, P., C. Sahlberg, P. Huhtala, T. Hurskainen, I. Thesleff, and K. Tryggvason.** 1992. Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. J. Biol. Chem. **267**:7856–7862.
34. **Reponen, P., C. Sahlberg, C. Munaut, I. Thesleff, and K. Tryggvason.** 1994. High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. J. Cell Biol. **124**:1091–1102.
35. **Riechmann, V., I. van Cruchten, and F. Sablitzky.** 1994. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucleic Acids Res. **22**:749–755.
36. **Roskelley, C. D., P. Y. Desprez, and M. J. Bissell.** 1994. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proc. Natl. Acad. Sci. USA **91**:12378–12382.
37. **Schmidhauser, C., M. J. Bissell, C. A. Myers, and G. F. Casperson.** 1990. Extracellular matrix and hormones transcriptionally regulate bovine beta-casein 5′ sequences in stably transfected mouse mammary cells. Proc. Natl. Acad. Sci. USA **87**:9118–9122.

37a. **Shalaby, R., and G. Colbern (California Pacific Medical Center).** Personal communication.

38. **Stetler-Stevenson, W. G.** 1990. Type IV collagenases in tumor invasion and metastasis. Cancer Metastasis Rev. **9**:289–303.
39. **Streuli, C. H., C. Schmidhauser, N. Bailey, P. Yurchenco, A. P. Skubitz, C. Roskelley, and M. J. Bissell.** 1995. Laminin mediates tissue-specific gene expression in mammary epithelia. J. Cell Biol. **129**:591–603.
40. **Sun, X. H.** 1994. Constitutive expression of the Id1 gene impairs mouse B cell development. Cell **79**:893–900.
41. **Talhouk, R. S., J. R. Chin, E. N. Unemori, Z. Werb, and M. J. Bissell.** 1991. Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture. Development **112**:439–449.
42. **Taub, M., Y. Wang, T. M. Szczesny, and H. K. Kleinman.** 1990. Epidermal growth factor or transforming growth factor α is required for kidney tubulogenesis in Matrigel cultures in serum free medium. Proc. Natl. Acad. Sci. USA **87**:4002–4006.
43. **Thompson, E. W., S. Paik, N. Brunner, C. L. Sommers, G. Zugmaier, R. Clarke, T. B. Shima, J. Torri, S. Donahue, M. E. Lippman, G. R. Martin, and R. B. Dickson.** 1992. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J. Cell. Physiol. **150**:534–544.
44. **Traurig, H. H.** 1967. Cell proliferation in the mammary gland during late pregnancy and lactation. Anat. Rec. **157**:489–504.
45. **Traurig, H. H.** 1967. A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. Anat. Rec. **159**:239–248.
46. **Wang, S., L. J. Counterman, and S. Z. Haslam.** 1990. Progesterone action in normal mouse mammary gland. Endocrinology **127**:2183–2189.
47. **Weintraub, H., R. Davis, S. J. Tapscott, M. Thayer, R. Krause, R. Benezra,**

**T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, et al.** 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science **251**:761–766.

48. **Wernert, N., F. Gilles, V. Fafeur, F. Bouali, M. B. Raes, C. Pyke, T. Dupressoir, G. Seitz, B. Vandenbunder, and D. Stehelin.** 1994. Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. Cancer Res. **54**: 5683–5688.
49. **Witty, J. P., J. H. Wright, and L. M. Matrisian.** 1995. Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. Mol. Biol. Cell **6**:1287–1303.
50. **Zhang, R. D., I. J. Fidler, and J. E. Price.** 1991. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. Invasion Metastasis **11**:204–215.

# MINA J. BISSELL
Distinguished Scientist
Lawrence Berkeley National Laboratory
One Cyclotron Rd., 83-101
Berkeley, CA 94720
mjbissell@lbl.gov


**EDUCATION**

| | | |
|---|---|---|
| Chemistry Transferred | Bryn Mawr College | 1959 – 1961 |
| B.A. (Honors) Chemistry | Radcliffe/Harvard College | 1961 – 1963 |
| M.A. Bacteriology and Biochemistry | Harvard University Medical School | 1963 – 1964 |
| Ph.D. Microbiology and Molecular Genetics | Harvard University Medical School | 1964 – 1969 |

**RESEARCH AND PROFESSIONAL EXPERIENCE:**

**Research Experience and Employment:**
Milton Fellow, Harvard Univ (1969–70); American Cancer Society Fellow (1970–72); Staff Biochemist (1972–76); Senior Staff, LBNL (1976–present); Faculty, Comparative Biochemistry (1979–present); Visiting Wellcome Prof., Kettering Inst., Univ. of Cincinnati Medical School (1986–88); Director, Cell & Molecular Biology Division, LBNL (Jan. 1988–92); Director, Life Sciences Division (includes Cell & Molecular Biology Division), LBNL (1992–2002); Distinguished Scientist (Nov. 2002-present); Senior Advisor to the Laboratory Director on Biology (Nov. 2002-present).

**Awards and Honors (selected):**
Medal for Top High School Student in the Country, Iran (1958); Medal of Amer. Inst. of Chemists for Top Chemistry Student at Radcliffe College (1962); Fogarty Senior Fellow (London, 1983–84); First Joseph Sadusk Award for Breast Cancer Research (1985); Guggenheim Fellow (Paris, 1992-93); ASCB Women in Cell Biology Career Recognition Award (1993); Elected AAAS Fellow (1994); E.O. Lawrence Award, US Dept. of Energy (1996); President, American Society of Cell Biology (ASCB,1997); Elected, Inst. of Medicine of the National Acad. of Sciences (1997); Exceptional Service Award, OBER, US Dept. of Energy (1997); Mellon Award, University of Pittsburgh (1998); Eli Lily/Clowes Award of the American Association for Cancer Research (AACR;1999); President, Int'l Society of Differentiation (ISD; 2000–2002); Honorary Doctorate, Pierre & Marie Curie University, Paris, France (2001); Innovator Award in Breast Cancer, US Department of Defense (2002); Elected to the American Academy of Arts and Sciences (2002); Komen Foundation Brinker Award (2003); More than 40 distinguished & named lectures.

**National & International Committees and Review Boards (selected):**
NIH Molecular Cytology Study Section (1981-85); NIH Gerontology & Geriatrics Review Study Section (1987–89); NIH Pathology B Study Section (1989–92); Board of Directors, Gordon Conferences (1993–98); Chair, Gordon Research Conference, Biological Structure and Gene Expression (1993); Secretary of Energy's Advisory Committee BERAC (1995–1999); Chair, BERAC Subcommittee on Application of Genome and Structural Biology (1995); Chair, Keystone Meeting on Breast and Prostate Cancer, Taos, NM (1996); Chair, NASA Committee on the Role of Animal Research in Space (1996–97); Integration Panel, U.S. Army Breast Cancer Research Program (1995–2003); NIH and NCI Panel on "Preclinical Models of Cancer" (1997-98); Howard Hughes Medical Inst. Evaluation Panel, Washington, DC (1997/1999); Board of Directors, American Association for Cancer Research (1999–2001);U.S. Representative to Council of Scientists, Human Frontier Science Program, Strasbourg, France (1998–2002); Advisory Committee, Burroughs Wellcome Fund's Career Awards in the Biomedical Sciences program (1998–2002); Human Rights Committee of National Academy of Sciences (1999–present); Advisory Board, Univ. Chicago Cancer Research Center (1998–present); Institute of Defense Analysis, Defense Science Study Group, Alexandria, VA (2000–present); AACR Science Policy and Legislative Affairs Committee (2001–2004); External Advisory Board, Institute for Molecular and Cell Biology, Porto, Portugal (IBMC) (1999–present); External Scientific Advisory Committee for the MIT Center for Environmental Health Sciences (2002–present.); Science Advisory Committee, Breakthrough Breast Cancer, London, UK (2002-present).

**Ass ciate Editor & Editorial Boards (current only):**
*In Vitro Cellular and Developmental Biology* (1990– ); *Journal of Cellular Biochemistry* (1990– ); *Molecular Carcinogenesis* (1993– ); *Cancer Research* (1994–; Senior Assoc. Editor, 2000- ); *The Breast Journal* (1994– ); *Cell Structure and Function* (1994– ); *Journal of Mammary Gland Biology* (1995– ); *Journal of Experimental Therapeutics* and Oncology (1995– ); *Molecular Medicine* (1997– ); *Breast Cancer Research* (1999– ; Senior Editor: 2003- ); *International Journal of Cancer* (1999– ); *The FASEB Journal* (2002– ).

**Patent Pending:**
IB–JIB-1791 A Suprabasal Breast Cell Line with Stem Cell Properties (with Olé William Petersen et al.)

**Patents Issued:**
United States Patent # 5,846,536: Restoration of Normal Phenotype in Cancer Cells
United States Patent # 6,123,941: Method for Restoration of Normal Phenotype in Cancer Cells
United States Patent # 6,287,790: Utilization of Nuclear Structural Proteins for Targeted Therapy and Detection of Proliferative and Differentiation Disorders

**Lectures (2002-Present):** *Plenary and named lectures are marked with an asterisk.*

**2002**
*Keystone Symposium, Biological Response to the Extracellular Matrix, The Matrix in Development, Banff, Canada
*Excellence in Cancer Research Seminar Series, Cross Cancer Institute, Edmonton, Alberta, Canada
*Bioengineering and Environmental Health & Chemistry Department, MIT, Boston, MA (Harris Lecture)
*Matrix and Morphogenesis: Celebrating Elizabeth Hay, Boston, MA
*International Society of Differentiation, Lyon, France (President's Lecture)
*DOD, Era of Hope, Breast Cancer Research Program Meeting, Orlando, FL (Innovation Lecture)
*Van Andel Institute, Grand Rapids, MI
*AACR (Proteases, Extracellular Matrix, and Cancer) Hilton Head, SC (Keynote Lecture)
*International Biomed Society, Stanford, CA

**Others include**: City of Hope, Los Angeles, CA; UCSF, Department of Surgery, San Francisco, CA; University of California, Los Angeles, Molecular Biology Institute, Graduate Program Seminar, Los Angeles, CA; Thomas Jefferson University, Kimmel Cancer Center, Philadelphia, PA; Duke University Medical Center, Signal Transduction Colloquium, Durham, NC Tufts University Medical School, Department of Anatomy and Cellular Biology, Boston, MA; Boston University, Boston, MA Biogen, Cambridge, MA; Morehouse College, Division of Science and Mathematics, Atlanta, GA; Emory University, Atlanta, Department of Cell Biology, GA; Whitehead Institute, Cambridge, MA; Harvard Medical School, Vascular Biology Seminar, Department of Pathology, Boston, MA; University of California, Davis, Medical Cancer Center, Sacramento, CA Oakland Outreach Program, Oakland, CA; Breast Cancer Foundation's Think Tank, Santa Barbara, CA; AstraZeneca, Boston, MA; Becton Technologies, RTP, North Carolina

**2003**

*Second Timberline Symposium on Epithelial Biology, My Hood, Oregon
*American Society of Investigative Pathology Conference, San Diego, CA
*Northwestern University, Chicago, IL (Mayberry Lecture)
*Danish Society for Cancer Research, Copenhagen, Denmark, Bristol (Myers Squibb Lecture)
*Gordon Research Conference: Mammary Gland Meeting, Bristol RI
*Gordon Research Conference: Cell Contact and Adhesion, Andover, NH (Keynote session)
*The Cancer Institute of New Jersey, Princeton, NJ
*University of Illinois, in Chicago, Chicago, IL (Krakower Lecture)
*Mahajani Symposium, San Diego, CA
*Epithelial-Mesenchymal Transitions Conference, Queensland, Australia
*University of California, Berkeley (BISC Distinguished Lecture)
*San Antonio Breast Cancer Conference (Komen Foundation, Brinker Award)

**Others include**: Caltech, Pasadena, CA; UCSF, Cancer Center, San Francisco, CA; Salk Institute, San Diego, CA University of California, San Diego, CA; University of California, Santa Cruz, CA; Huntsman Cancer Institute, Salt Lake City, Utah; Endocrine Therapy Conference, Boston, MA; Tularik Inc. San Francisco, CA; Ernest Gallo Clinic and Research Center, Emeryville, CA; Cell Genesys, San Francisco, CA; California Breast Cancer Research Program, San Diego, CA ComBio 2003 Meeting, Melbourne, Australia.

**Publications (selected since 1995; total 236):**


131. Boudreau N, Sympson CJ, Werb Z and **Bissell MJ**. (1995) Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 267:891-893..
135. Lin CQ, Dempsey P, Coffey C and **Bissell MJ**. (1995) Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF-a in mouse mammary epithelial cells: Studies in culture and in transgenic mice. *J. Cell Biol* 129(4):1115-1126.
137. Rønnov-Jessen L, Petersen OW, Kotelianski V and **Bissell MJ**. (1995) The origin of the myofibroblasts in breast cancer: Recapitulation of tumor environment in culture unravels diversity and implicates coverted fibroblasts and recruited smooth muscle cells. *J Clinical Investigation* 95:859-873.
139. Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz A and **Bissell MJ**. (1995) Laminin mediates tissue-specific gene expression in mammary epithelia. *J Cell Biol* 129:591-603.
154. Rønnov-Jessen L, Petersen OW and **Bissell MJ**. (1996) Cellular changes involved in conversion of normal to malignant breast: The importance of the stromal reaction. *Physiol Revs* 76:69-125.
159. Ashkenas J, Muschler J and **Bissell MJ**. (1997) The extracellular matrix in epithelial biology: Shared molecules and common themes in distant phyla. *Developmental Biol* 180:433-444.
162. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z and **Bissell MJ**. (1997) Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 139:1861-1872.
163. Lochter A, Srebrow A, Sympson CJ, Terracio N, Werb Z and **Bissell MJ**. (1997) Misregulation of stromelysin-1 expression in mouse mammary tumor cells accompanies acquisition of stromelysin-1-dependent invasive properties. *J Biol Chem* 272:5007-5015.
165. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C and **Bissell MJ**. (1997) Reversion of the malignant phenotype of human breast cells in three-dimensional culture and *in vivo* using integrin blocking antibodies. *J Cell Biol* 137:231-246 (cover feature).
171. Hirai Y, Lochter A, Galosy S, Koshida S, and **Bissell MJ**. (1998) Epimorphin, not hepatocyte growth factor or epidermal growth factor, functions as a morphoregulatory molecule for mammary epithelial cells. *J Cell Biol* 140:159-169.
172. Lelièvre SA, Weaver VM, Nickerson JA, Larabell CA, Bhaumik A, Petersen OW and MJ **Bissell MJ**. (1998) Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus. *Proc Natl Acad Sci USA* 95:14711-14716.
175. Myers CA, Schmidhauser C, Mellentin-Michelotti J, Fragoso G, Roskelley CD, Casperson G, Mossi R, Pujuguet P, Hager G and **Bissell MJ**. (1998) Characterization of BCE-1: A transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. *Mol Cell Biol* 18(4):2184-2195.
180. Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P, Lupu R and **Bissell MJ**. (1998) Reciprocal interactions between β1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology. *Proc Natl Acad Sci USA* 95:14821-14826.
182. **Bissell MJ**. (1999) Tumor plasticity allows vasculogenic mimicry, a novel form of angiogenic switch: A rose by any other name? *Am J Pathol* 155(3):675-9.
184. **Bissell MJ**, Weaver VM, Lelièvre SA, Wang F, Petersen OW and Schmeichel KL. (1999) Tissue structure, nuclear organization and gene expression in normal and malignant breast. *Cancer Res.* 59:1757s-1764s.
185. Lochter A, Navre M, Werb Z and **Bissell MJ**. (1999) β1 and β2 integrins mediate invasive activity of mouse mammary carcinoma cells through regulation of stromelysin-1 expression. *Mol Biol Cell* 10:271-282.
188. Muschler J, Lochter A, Roskelley CR, Yurchenco P and **Bissell MJ**. (1999) Division of labor among the α6ß4 integrins and an E3 laminin receptor to signal morphogenesis and ß-casein expression in mammary epithelial cells. *Mol Biol Cell* 10(9):2817-28.
189. Péchoux C, Gudjonsson T, Rønnov-Jessen L, **Bissell MJ** and Petersen OW. (1999) Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. *Develop Biol* 206:88-99.
191. Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier J-P, Gray J, Pinkel D, **Bissell MJ**, and Werb Z. (1999) The stromal proteinase MMP-3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98(2):137-146.
195. Pujuguet P, Simian M, Liaw J, Timpl R, Werb Z and **Bissell MJ**. (2000) Nidogen-1 regulates laminin-1-dependent mammary-specific gene expression. *J Cell Sci* 113 (Pt 5):849-858 (cover feature).
197. Chen M, Schmeichel K, Mian IS, Lelièvre SA, Petersen OW and **Bissell MJ**. (2000) AZU-1: A candidate breast tumor suppressor and biomarker for tumorigenic reversion. *Mol Biol Cell* 11(4):1357-1367.

200. Hirai Y, Radisky D, Boudreau R, Simian M, Stevens M, Oka Y, Takebe K, Niwa S and **Bissell MJ**. (2001) Epimorphin mediates mammary luminal morphogenesis through control of C/EBPβ. *J Cell Biol* 153(4):785-794.
201. Simian M, Hirai Y, Navre M, Werb Z, Lochter A and **Bissell MJ**. (2001) The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development* 28:3117-3131.
202. Muthuswamy SK, Li D, Lelièvre SA, **Bissell MJ** and Brugge JS. (2001) Erbβ2, but not Erbβ1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nature Cell Biol* 3(9):785-792.
203. **Bissell MJ** and Radisky D. Putting tumours in context. (2001) *Nature Reviews (Cancer)* 1:48-54.
208. Gudjonsson T, Rønnov-Jessen L, Villadsen R, **Bissell MJ** and Petersen OW. (2002) Normal and tumor-derived myoepithelial cells differ in their ability to signal to luminal breast epithelial cells for polarity and basement membrane deposition. *J Cell Science* 115(1):39-50.
211. Gudjonsson T, Villadsen R, Nielsen HL, Rønnov-Jensen L, **Bissell MJ**, and Petersen OW. (2002) Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. *Genes & Development* 16(6):693-706.
213. Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA and **Bissell MJ**. (2002) Phenotypic Reversion or Death of Cancer Cells by Altering Signaling Pathways in Three-Dimensional Contexts. *J Nat'l Cancer Inst* 94(19):1494-1503.
214. Weaver VM, Lelièvre SA, Lakins JN, Chrenek MA, Jones JCR, Giancotti F, Werb Z and **Bissell MJ**. (2002) β4 Integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell* 2:205-216; ***News & Views, Nature 419:790-791 (2002). Minireview (Cell) 111:923-925* (2002).**
215. Muschler J, Levy D, Boudreau R, Henry M, Campbell K and **Bissell MJ**. (2002) A role for dystroglycan in epithelial polarization: Loss of function in breast tumor cells. *Cancer Research* 62:7102-7109.
216. **Bissell MJ**, Radisky D, Rizki A, Weaver, VM and Petersen, OW. (2002) The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation* 70(9-10):537-46.
217. Petersen OW, Nielsen HL, Gudjonsson T, Villadsen R, Rank F, Niebuhr E, **Bissell MJ** and Rønnov-Jessen L. (2003) Epithelial to Mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol* 162:2:391-402.
218. **Bissell MJ**, Mian IS, Radisky D and Turley E. (2003) Tissue-specificity: Structural cues allow diverse phenotypes from a constant genotype. In: Origination of Organismal Form: Beyond the Gene in Developmental and Evolutionary Biology, Müller GB and Newman SA (Eds.). The Vienna Series in Theoretical Biology. *MIT Press* 7:103-117.
219. **Bissell MJ** and Bilder D. (2003) COMMENTARY: Polarity determination in breast tissue: desmosomal adhesion, myoepithelial cells, and laminin 1. *Breast Cancer Res* 5:2:117-119.
220. Anders M, Hansen R, Ding R-X, Waldman FW, Rauen K, **Bissell MJ**, and Korn WM. (2003) Disruption of 3D tissue integrity facilitates adenovirus infection by deregulating the coxsackievirus and adenovirus receptor. *Proc Natl Acad Sci* 100:4:1943-1948.
221. Bhatacharyya C, Grate LR, Rizki A, Radisky D, Molina FJ, Jordan MI, **Bissell MJ**, Mian IS. (2003) Simultaneous relevant feature identification and classification in high-dimensional spaces: Application to molecular profiling data. *Signal Processing* 83:4:729-743.
222. Schmeichel, KL and **Bissell MJ**. (2003) Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci.* 116:2377-2388.
223. Novaro V, Roskelley C and **Bissell MJ**. (2003) Collagen-IV or laminin-1 together with lactogenic hormones regulate estrogen receptor-alpha expression and function in mouse mammary epithelial cells. *J Cell Science* 116(14) 2975-2986.
224. Gudjonsson T, Ronnov-Jessen L, Villadsen R, **Bissell MJ**, Petersen OW. (2003) To create the correct microenvironment: three-dimensional heterotypic collagen assays for human breast epithelial morphogenesis and neoplasia. *Methods* Jul;30(3):247-55.
225. Radisky, DC, Hirai, Y and **Bissell MJ.** (2003) Delivering the message: epimorphin and mammary epithelial morphogenesis. *Trends Cell Biol* 13(8):426-34.
226. Park, CC, Henshall-Powell, RL, Erickson, AC, Talhouk, R, Parvin, B, **Bissell MJ** and Barcellos-Hoff MH. Ionizing radiation induces heritable disruption of epithelial cell interactions. *Proc Natl Acad Sci* 100:19:10728-10733.
227. Wiseman BS, Sternlicht MD, Lund LR, Alexander CM, Mott J, **Bissell MJ**, Soloway P, Itohara S and Werb Z. (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *Journal of Cell Biology* 162 (6):1123-1133.
228. Fata JE, Werb, Z and **Bissell MJ.** (2003) Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res* Review 6:1-11.
229. Petersen OW, Gudjonsson T, Villadsen R, **Bissell MJ**, Ronnov-Jessen L. (2003) Epithelial progenitor cell lines as models of normal breast morphogenesis and neoplasia. *Cell Prolif.* Oct;36 Suppl 1:33-44. Review.
230. Park C, Zhang H, Peng M, **Bissell MJ**. (2003) Cell-ECM mediated radiation response in breast cancer: beta1 integrin as a potential molecular target. *Int J Radiat Oncol Biol Phy* Oct 1;57(2 Suppl):S161.
231. Kenny, PA and **Bissell MJ**. (2003) Tumor reversion: Correction of malignant behavior by microenvironmental cues. *Int J Cancer* Review 107(5):588-695.
232. **Bissell MJ**, Rizki A, Mian IS (2003) Tissue architecture: the ultimate regulator of breast epithelial function. *Curr Opin Cell Biol* Dec;15(6):753-62.
233. Come SE, Buzdar AU, Arteaga CL, **Bissell MJ**, Brown MA, Ellis MJ, Goss PE, Green JE, Ingle JN, Lee AV, Medina D, Nicholson RI, Santen RJ, Schiff R, Hart CS. Proceedings of the third international conference on recent advances and future directions in endocrine manipulation of breast cancer: conference summary statement. *Clin Cancer Re.* 2004 Jan 1;10(1 Pt 2):327S-30S.
234. Novaro V, Radisky DC, Ramos Castro NE, Weisz A, **Bissell MJ**. (2004) Malignant mammary cells acquire independence from extracellular context for regulation of estrogen receptor alpha. *Clin Cancer Res* Jan 1;10(1 Pt 2):402S-9S.
235. Boudreau N, Myers C, **Bissell MJ**. (2004) From laminin to lamin: regulation of tissue-specific gene expression by the ECM. *Trends Cell Biol* 1995 Jan;5(1):1-4.
236. Liu H, Radisky DC, Wang F and **Bissell MJ**. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. *J Cell Biol* (In press -2004).

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

| NAME | POSITION TITLE |
|---|---|
| Muschler, John L. | Associate Scientist |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| University of Illinois, Urbana, IL | B.S. | 1986 | Chemistry |
| University of Illinois, Urbana, IL | Ph.D. | 1993 | Biochemistry |

**Professional Experience:**

1984-85 **Research Associate**, U.S.D.A. laboratory, University of Illinois, Urbana.
1986-87. **Research Associate**, Department of Biochemistry, University of Illinois.
1987 **Teaching Assistant**, Chemistry, University of Illinois, Urbana.
1989 **Teaching Assistant**, Biochemistry, University of Illinois, Urbana.
1993-1995 **Postdoctoral Research Fellow**
Laboratoire de Biologie Moleculaire,
Pasteur Institute, Paris, France
1995-2000 **Postdoctoral Research Fellow**
Life Science Division
Lawrence Berkeley National Laboratory, Berkeley, CA.
2000-2002 **Scientist**
Life Science Division,
Lawrence Berkeley National Laboratory, Berkeley, CA.
2002-present **Associate Scientist**
California Pacific Medical Center Research Institute,
San Francisco, CA.

2003 Grant Reviewer for the Department of Defense Breast Cancer Research Program.

**Honors and Fellowships:**

1989 University of Illinois Biotechnology Center Award.
1993-1995 Long Term Fellowship, European Molecular Biology Organization (EMBO).
1995-1997 Individual National Research Service Award.
1998-2000 Postdoctoral Fellowship, Department of Defense Breast Cancer Research Program.
2000-2003 New Investigator Award, California Breast Cancer Research Program.
2001-2004 IDEA Grant, Department of Defense Breast Cancer Research Program.

**Publications:**

Henry, I., Forlani, S., Vaillant, S., Muschler, J., Choulika, A. and Nicolas, J.-F. (1999) "LagoZ and LagZ, two genes derived from LacZ by depletion of CpG dinucleotides for the study of epigenetic controls". C. R. Acad. Sci., Paris. 322: 1-9.

Muschler, J., Lochter, A., Roskelley, C.D., Yurchenco, P. and Bissell, M.J. (1999). Division of labor among the α6β4 integrin, β1 integrins, and an E3 laminin receptor to signal morphogenesis and β-casein expression in mammary epithelial cells. Mol. Biol. Cell 10: 2817-2828.

McDonald KA., Muschler J., and Horwitz AF. (1998). Immunopurification of a sarcomeric junctional protein complex containing GAPDH. Exp. Cell Res. 243: 305-18

Lochter A., Galosy S., Muschler J., Freedman N., Werb Z. and Bissell MJ. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J. Cell Biol. 139:1861-72

Ashkenas J., Muschler J. and Bissell MJ. (1996). The extracellular matrix in epithelial biology: shared molecules and common themes in distant phyla. Dev. Biol. 180:433-44

Sastry SK., Lakonishok M., Thomas DA., Muschler J. and Horwitz AF. (1996). Integrin alpha subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. J. Cell Biol. 133:169-84

Lakonishok M., Muschler J. and Horwitz AF. (1992). The α5β1 integrin associates with a dystrophin-containing lattice during muscle development. Dev. Biol. 152:209-20

Bronner-Fraser M., Artinger M., Muschler J. and Horwitz AF. (1992). Developmentally regulated expression of α6 integrin in avian embryos. Development 115:197-211

Bao ZZ., Muschler J. and Horwitz AF. (1992). LBL, a novel, developmentally regulated, laminin-binding lectin. J. Biol. Chem. 267:4974-80

Muschler JL. and Horwitz AF. (1991). Down-regulation of the chicken α5β1 integrin fibronectin receptor during development. Development 113:327-37

**Recent Abstracts:**

Weir, L., Itahana, Y., Campbell, K., Bissell, M.J., Muschler, J. Dystroglycan is essential for the response of mammary epithelial cells to laminin-1 and HSPGs. American Society for Cell Biology Annual Meeting. (Dec., 2003). San Francisco, CA.

Singh, J, Itahana, Y, Campbell, K., Muschler J. Proteolytic enzymes and altered glycosylation modulate dystroglycan function in carcinoma cells. American Society for Cell Biology Annual Meeting. (Dec., 2003). San Francisco, CA.

Mott, J., Muschler, J., Bissell, M.J. Phenotypic Reversion of the Human Breast Tumor Cell Line T4-2 Correlates with a Decrease in MMP-9 Production. American Society for Cell Biology Annual Meeting. (Dec., 2003). San Francisco, CA.

Muschler, J., Campbell, K. and Bissell, M.J. Restoration of dystroglycan function as a novel target in breast cancer therapy". The Era of Hope Meeting of the Department of Defense Beast Cancer Research Program. (Sept., 2002). Orlando, FL.

Muschler, J., Levy, D., Henry, M., Campbell, K. and Bissell, M.J. Loss of dystroglycan in breast tumor cells. American Society for Cell Biology Annual Meeting. (Dec., 2001). Washington D.C.

**Invited Lectures and Symposia:**

"Restoration of DG Function as a novel target in breast cancer therapy". The Era of Hope Meeting of the Department of Defense Beast Cancer Research Program. (Sept., 2002). Orlando, FL. 2002

"Linking dystroglycan, polarity and tumor suppression in breast epithelial cells." University of California at San

Customer Number
08076

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bissell et al.

| | | | |
|---|---|---|---|
| Serial No.: | 09/652,493 | Group No.: | 1642 |
| Filed: | August 31, 2000 | Examiner: | Yu |

Entitled: DESIGN OF NOVEL DRUG SCREENS BASED ON THE NEWLY FOUND ROLE OF DYSTROGLYCAN PROTEOLYSIS IN TUMOR CELL GROWTH

# APPEAL BRIEF UNDER 37 CFR 1.192

Commissioner for Patents
Mail Stop Appeal Brief – Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)**

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: Feb 10, 2004 By: Shawn Kay Reinemann

Dear Sir:

Applicant hereby submits this brief in support of an appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner. This brief is filed pursuant to Applicants' Notice of Appeal filed with the USPTO by facsimile transmission on December 19, 2003. Appropriate transmittal and fee papers are transmitted herewith.

CONTENTS Page



## 1. Real Party in Interest

The real parties in interest are The Regents of the University of California and the United States Government.

## 2. Related Appeals and Interferences

There are no known related appeals (or interferences).

## 3. Status of Claims

Pursuant to Final Office Action dated July 24, 2003, claims 1-8, 22-24, 29 and 30 are pending in the application, and these claims are all finally rejected. Claims 9-21 and 25 were earlier cancelled without prejudice pursuant to a restriction requirement. Claims 29 and 30 were added by amendment dated August 15, 2002.

## 4. Status of Amendments

All Amendments have been entered.

## 5. Summary of Invention

The present invention is directed to the finding by applicants that a previously unrecognized fragment of α-dystroglycan, having an Mr of 120-130kD, is shed from cells having tumorigenic properties.

Dystroglycan was originally identified in skeletal muscle as a component of the dystrophin-glycoprotein complex. It is composed of α- and β-subunits which are encoded by a single gene, and cleaved into two proteins by posttranslational processing. Dystroglycan is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to a transmembrane glycoprotein, β-dystroglycan. Normal α dystroglycan has a molecular weight of about 180 kD. Dystroglycan spans the sarcolemma (muscle fiber membrane) and its known function is to provide a connection between the extracellular matrix and the cytoskeleton. Dystrophin deficiency causes a drastic reduction of the dystroglycan complex in the sarcolemma and, thus, loss of linkage between the subsarcolemmal cytoskeleton and the extracellular matrix, eventually leading to muscle cell death in Duchenne muscular dystrophy.

The present application extends previous research from the laboratory of the present inventors, as illustrated in Attachment 1, Lelievre et al. "Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus," Proc. Acad. Sci. 95:14711-14716 (Dec. 1998). That is, investigations have shown certain links between tumorgenic phenotypes and properties

on the *outside* of cells, rather than genetic changes on the inside of cells. The present work is consistent with that theme in that the conventional role of dystroglycan involves interaction with the extracellular matrix, rather than a role in growth control. Specifically, the present work is directed to a newly found behavior of α dystroglycan in cancer cells. Fig. 1 illustrates this by showing an SCg6 mammary carcinoma cell supernatant having a distinct band in the 120-130kD region, representing a shed α-dystroglycan fragment. (SCg6 is known to be a tumorigenic cell line. See Attachment 2.). To detect the present 120-130kD fragment, one may use a cell medium assay (using the disclosed antibody IIH6 on p. 16 of the Specification) or use a cellular assay to determine the existence and extent of this shedding. The degree to which the α-dystroglycan on a cell surface has been cleaved and shed into the medium correlates with the tumorigenicity of the cell. In Fig. 2, it is shown that 5 of 8 tumor cell lines tested lacked detectable cell surface α-dystroglycan.

Accordingly, the present claims are directed to methods of detecting the shedding of the 120-130 kD fragment.

**6. Issues**

The presently outstanding final office action sets forth one rejection, raising the following issues:

A. Are Claims 1-8, 22-23, 29 and 30 enabled by the specification?

B. Does Dr. Campisi's Declaration constitute objective evidence that the specification contains sufficient guidance to enable one of ordinary skill in the art to make and use the claimed invention(s)?

C. Is undue experimentation required for assessing whether detection of α dystroglycan fragments correlates with tumor growth and tumorigenicity because it requires analysis of a large quantity of clinical samples to determine whether or not shedding of said fragments occurs at all in vivo and detection of said fragments is indicative of tumor growth and potential tumorigenicity? Since a large number of clinical samples is required, is it proper to require Applicants to submit "evidence showing that detection of α dystroglycan fragment in blood is correlated with tumor growth and/or potential tumorigenicity in vivo in order to obviate this rejection"? (See Final Office Action, page 3, end of first paragraph.)

**7. Grouping of Claims**

Insofar as the rejection is directed to a lack of "objective evidence" that shedding of the present α dystroglycan fragment is correlated to tumorigenicity all of the claims on appeal stand or fall together because they speak to shedding of the fragment in vivo. Claim 1 contains the phrase "whereby the presence of the fragment indicates higher potential tumorigenicity." Claim 22 contains the phrase "positively correlated with tumor cell growth." For purposes of the present appeal, these phrases are believed to raise the same issue.

**8. Arguments**

**A. The claims are enabled.**

Firstly, the question of enablement is directed to whether or not undue experimentation is required to make and use the claimed invention. There is no doubt in

this case that one of ordinary skill can make the presently claimed invention. What is in dispute is whether or not the use of the invention will have the beneficial result referred to in the claims, i.e. assessment of tumorigenic potential. The present method may not be a fully developed method immediately suitable for clinical use, but it has sufficient utility to meet the requirements of 35 USC 101. The Examiner doubts the assertions made throughout the specification by the inventors, both Ph.D. scientists doing full time research in this field. See inventors' CV's attachment 3 and attachment 4.

As stated in MPEP 2164.04,

> In order to make a[n enablement] rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, sought to 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, **unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.** Assuming that sufficient reason for such doubt exists, a rejection for failure to teach how to make and/or use will be proper on that basis. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370. (emphasis supplied in bold).

In this case, no reason to doubt Applicants' assertions has been presented. Arguments that there may be cases where normal cells shed the α-dystroglycan fragment are pure speculation.

**B. The Declaration of Dr. Judith Campisi provides objective evidence that the claims are enabled.**

Dr. Judith Campisi is a Senior Staff Scientist at Lawrence Berkeley National Laboratory. She has no interest in the present patent application other than common employment with the present inventors. In her declaration of May 5, 2003, she states:

> 5. In my experience, a number of *in vitro* cell culture models are generally recognized in the art as correlating to *in vivo* conditions of tumorigenicity or tumorigenic potential. The three dimensional basement membrane assay, employed in the present application, is especially well regarded as predictive of *in vivo* cell growth behavior of tumor cells. Furthermore, the present specification describes nude mouse experiments that further confirm the correlation between the present *in vitro* assay and *in vivo* results. The present specification provides working examples describing the detection of the 120-130 kD α dystroglycan fragment in cell culture medium. It further provides results from a reasonable number of cell lines to show the correlation between shedding of this fragment *in vitro* and potential tumorigenicity, as represented in Fig. 2 of the specification. In this particular case, I believe that it is scientifically credible and plausible to extrapolate the detection of a shed dystroglycan fragment found in cell culture medium to the ability to find that same fragment, using similar techniques, in the blood or other tissue of a living animal, including a human. Furthermore, the teachings of the present specification support this expectation. I have reviewed the inventors' follow up paper, Muschler et al., "A Role for Dystroglycan in Epithelial Polarization: Loss of Function in Breast Tumor Cells," *Cancer Research* 62:7102-7109 (Dec. 2001), and note that they have obtained *in vivo* data in nude mice that correlate with their *in vitro* work. The nude mouse model is generally accepted as a model predictive of human tumor cell behavior.
>
> 6. I have reviewed the inventor's conclusions in the specification, in particular the discussion of the shedding of α dystroglycan fragments in hyperplasia and tumor cell growth. Specifically, I have read the following:

"Because α and β dystroglycan are translated as a single polypeptide, it was surprising that α dystroglycan was not detected on the cell surface of many cells when β dystroglycan was present. We concluded that, by some mechanism, α dystroglycan was being shed from the cell surface." (Page 11, first paragraph).

"We believe α dystroglycan shedding occurs principally in cells that are reorganizing and growing. Little of such activity occurs in adult tissues, except in cases like the normal processes of mammary gland development, and perhaps angiogenesis. However, such activity would occur on a large scale during hyperplasia or tumor cell growth and the accompanying angiogenesis. α Dystroglycan is shed in two forms, one which binds laminin and a smaller portion with no known binding activity. An assay that detects α dystroglycan proteolysis would be an assay for the detection of tissue re-organization and cell growth." (Page 13-14)

7. I believe that the weight of scientific evidence favors the statements quoted in Paragraph 6, rather than raising doubt as to the truth of these statements. The reference to "tissue re-organization and cell growth" also applies also to "potential tumorigenicity."

Thus, objective evidence has been provided for the method of using the presently claimed invention.

**C. Clinical trials or other in vivo data are not required under 35 USC 112 and are not necessary to show a reasonable expectation of success in this case.**

The "how to use" prong of the utility requirement does not require in vivo data for a biological invention. In accordance with MPEP 2164.02,

The issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" as used herein refers to the relationship between in vitro or in vivo animal model assays and a disclosed or a claimed method of use. An in vitro or in vivo animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If there is no correlation, then the examples do not constitute "working examples." In this regard, the issue of "correlation" is also dependent on

> the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that in vitro data did not support in vivo applications).
>
> Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an in vitro or in vivo animal model example. A rigorous or an invariable exact correlation is not required, as stated in Cross v. Iizuka, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):
>
> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed in vitro utility and an in vivo activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (Citations omitted).

Thus the "invitation" in the Final Office Action that the Applicants supply data showing detection of the shed fragment from in vivo experiments (in order to obtain allowance of the claims) is improper.

**9. Conclusion**

The present case involves a novel finding in the relationship between the cell membrance and the extracellular matrix. Modelling of in vivo cell behavior in an in vitro extracellular matrix model is accepted in the art (See Attachment 1, and Specification, page 2, first full paragraph. Specifically, the present inventors show that cleavage of a cellular adhesion molecule is correlated with increased tumorigenic potential in tests run on eight selected cell lines. The specification describes an assay that has an immediate utility in the laboratory as studying tumorigenic potential and may be extended to clinical use without undue experimentation.

Accordingly, the rejection of claims 1-8, 22-24, 29 and 30 under 35 USC 112 should be reversed and the present case passed to issuance.

Respectfully submitted,

Date: Feb 10 04

David J. Aston
Attorney for Applicant
Reg. No.: 28,051
Tel. No. (510) 486-7503
Lawrence Berkeley Laboratory
One Cyclotron Road
MS 90B0104
Berkeley, CA 94720

**10. Appendix: Claims on Appeal**

Claim 1. A method for measuring potential tumorigenicity of mammalian cells comprising:

a. providing a sample of medium surrounding cells, and

b. detecting the presence of a fragment of α-dystroglycan in medium, said fragment having an Mr of 120-130kD, whereby the presence of the fragment indicates higher potential tumorigenicity.

Claim 2. The method of claim 1, wherein said detecting comprises:

a. adding to said sample a material selected from the group consisting of a monoclonal antibody to α-dystroglycan and laminin, and

b. measuring the size of the α-dystroglycan fragment detected.

Claim 3. The method of claim 1, wherein said cells are human mammary epithelial cells.

Claim 4. The method of claim 1, wherein said medium is blood serum.

Claim 5. A method for measuring potential tumorigenicity of cells, comprising:

a. providing a sample of said cells, and

b. detecting the presence of α-dystroglycan on the surface of the cells, whereby the absence of α-dystroglycan indicates a higher potential for tumorigenicity.

Claim 6. The method of claim 5, wherein said detecting comprises:

a. adding to said sample a monoclonal antibody to α-dystroglycan, and

b. measuring the amount of labeled α-dystroglycan detected.

Claim 7. The method of claim 5, wherein said cells are human mammary epithelial cells.

Claim 8. The method of claim 5, wherein said detecting comprises measurement of the amount of α-dystroglycan relative to the amount of β-dystroglycan, wherein a relative decrease of α-dystroglycan indicates α-dystroglycan shedding and higher potential tumorigenicity.

Claims 9 – 21 (Withdrawn).

Claim 22. A method of assaying proteolysed α-dystroglycan fragments shed from a cell into blood serum comprising the steps of:

a. contacting a serum sample to be assayed with a labeled antibody specific for an α-dystroglycan fragment, and

b. assaying the amount of bound label, wherein said α-dystroglycan fragments bound to said labeled antibody are positively correlated with tumor cell growth.

Claim 23. The method of Claim 22, wherein the α-dystroglycan fragment is an approximately 120 kD fragment.

Claim 24. The method of Claim 22, wherein the α-dystroglycan fragment is an approximately 60 kD fragment.

Claims 25 – 28 (Withdrawn).

Claim 29. The method of claim 22, wherein said cell is an epithelial cell.

Claim 30. The method of claim 29, wherein said epithelial cell is a breast epithelial cell.

*Proc. Natl. Acad. Sci. USA*
Vol. 95, pp. 14711–14716, December 1998
Cell Biology

# Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus

SOPHIE A. LELIÈVRE*, VALERIE M. WEAVER*, JEFFREY A. NICKERSON†, CAROLYN A. LARABELL*, ANKAN BHAUMIK*, OLE W. PETERSEN‡, AND MINA J. BISSELL*§

*Lawrence Berkeley National Laboratory, Berkeley, CA 94720; †University of Massachusetts Medical School, Worcester, MA 01655; and ‡The Panum Institute, DK-2200 Copenhagen N, Denmark

*Communicated by Sheldon Penman, Massachusetts Institute of Technology, Cambridge, MA, September 30, 1998 (received for review August 14, 1998)*

**ABSTRACT** **What determines the nuclear organization within a cell and whether this organization itself can impose cellular function within a tissue remains unknown. To explore the relationship between nuclear organization and tissue architecture and function, we used a model of human mammary epithelial cell acinar morphogenesis. When cultured within a reconstituted basement membrane (rBM), HMT-3522 cells form polarized and growth-arrested tissue-like acini with a central lumen and deposit an endogenous BM. We show that rBM-induced morphogenesis is accompanied by relocalization of the nuclear matrix proteins NuMA, splicing factor SRm160, and cell cycle regulator Rb. These proteins had distinct distribution patterns specific for proliferation, growth arrest, and acini formation, whereas the distribution of the nuclear lamina protein, lamin B, remained unchanged. NuMA relocalized to foci, which coalesced into larger assemblies as morphogenesis progressed. Perturbation of histone acetylation in the acini by trichostatin A treatment altered chromatin structure, disrupted NuMA foci, and induced cell proliferation. Moreover, treatment of transiently permeabilized acini with a NuMA antibody led to the disruption of NuMA foci, alteration of histone acetylation, activation of metalloproteases, and breakdown of the endogenous BM. These results experimentally demonstrate a dynamic interaction between the extracellular matrix, nuclear organization, and tissue phenotype. They further show that rather than passively reflecting changes in gene expression, nuclear organization itself can modulate the cellular and tissue phenotype.**

The cell nucleus is organized by a nonchromatin internal structure referred to as the nuclear matrix (NM; refs. 1–3). Identified NM components include coiled-coil proteins (4), cell cycle regulators (5), tissue-specific transcription factors (6, 7), and RNA splicing factors (for review see ref. 2). Although splicing factors have been shown to redistribute during cellular differentiation (8, 9) and following the induction of gene expression (10), spatial distribution of nuclear components are thought to be the consequence of changes in gene expression (8, 10, 11). However, whether NM composition and structure may themselves affect gene expression and cellular function has not been examined.

To systematically study the effect of cell growth and tissue differentiation on nuclear organization, we used a reconstituted basement membrane (rBM)-directed model of mammary gland morphogenesis (12). The HMT-3522 human mammary epithelial cells (HMECs) were isolated from reduction mammoplasty and became immortalized in culture (13). When embedded within a rBM, these cells arrest growth, organize an endogenous BM, and form polarized acinus-like structures with vectorial secretion of sialomucin into a central lumen (12). We used this model to compare the nuclear organization of HMECs cultured on a plastic surface [two-dimensional (2D) monolayer] vs. a three-dimensional (3D) rBM. Nuclear organization was assessed by examining the distribution of the coiled-coil NM proteins lamin B (14) and NuMA (15), the cell cycle regulator Rb (p110Rb; ref. 5), and the splicing factor SRm160 (formerly known as B1C8; ref. 16). These proteins had distinct spatial distribution patterns specific for proliferation, growth arrest, and acini formation. Moreover, disruption of nuclear organization in acini by either perturbing histone acetylation or directly modifying the distribution of NM proteins altered the acinar phenotype.

We previously hypothesized (17) and thereafter provided evidence that the extracellular matrix (ECM) directs morphogenesis and gene expression in mammary epithelial cells (12, 18, 19). Here we show that a reciprocal relationship exists between the ECM and nuclear organization. These findings underscore a role for nuclear organization in regulation of gene expression and provide a possible framework for how cell–ECM interactions determine cell and tissue phenotype.

## MATERIALS AND METHODS

**Cell Culture.** HMT-3522 HMECs (S-1 passage-50 cells; ref. 13) were propagated in 2D cultures in chemically defined medium (12), and growth arrest was induced by removing epidermal growth factor (EGF) for 48 hr. Cultures were prepared by embedding single cells ($8.5 \times 10^5$ cells per ml of matrix) in rBM (Matrigel, Collaborative Research) or collagen-I matrix (Cellagen AC-5, ICN) in 4-well chamber slides (Nalge). These cultures were grown for 5–10 days. Growth arrest and morphogenesis were routinely observed by days 7–9.

**Antibodies and Inhibitors.** For Western blots and/or immunostaining, we used mAbs against type IV collagen (clone CIV, Dako), β-catenin (clone 14, Transduction Laboratories, Lexington, KY), SRm160 splicing factor (clone B1C8, 16), lamin B (clone 101-B7, Matritech, Cambridge, MA), NuMA (clone 204–41, Matritech, and clone B1C11, a gift from S. Penman, Massachusetts Institute of Technology, Cambridge, MA), and polyclonal antibodies (pAbs) against Ki-67 (Novo-Castra, Newcastle, U.K.), acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY), and p110Rb (Santa Cruz Biotechnology). For bioperturbation assays, we used mAbs against lamins A/C (clone 636, Novocastra, Newcastle, U.K.)

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Abbreviations: NM, nuclear matrix; BM, basement membrane; rBM, reconstituted BM; HMEC, human mammary epithelial cells; 2D and 3D, two and three dimensional; Rb, retinoblastoma protein; ECM, extracellular matrix; EGF, epidermal growth factor.

§To whom reprint requests should be addressed at: Lawrence Berkeley National Laboratory, Life Sciences Division, MS 83-101, Berkeley, CA 94720. e-mail: mjbissell@lbl.gov.

and NuMA (clone 22, Transduction Laboratories, Lexington, KY), in addition to B1C11 and 101-B7. Trichostatin A (Wako Chemicals, Richmond, VA) was used as an inhibitor of histone deacetylase (40 nM).

**Indirect Immunofluorescence.** Cells were permeabilized *in situ* (0.5% Triton X-100 in 100 mM NaCl/300 mM sucrose/10 mM Pipes, pH 6.8/5 mM MgCl2 containing 1 mM Pefabloc Sc (AEBSF) (Boehringer Manheim)/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml trypsin inhibitor type II/250 μM NaF), fixed in 2% paraformaldehyde, and immunostained as described (18). Human mammary tissue was snap-frozen in n-hexane and embedded in Tissue-Tek O.C.T. compound (Sakura Firetek, Torrance, CA); 5-μm sections were fixed in methanol and immunostained in accordance with human protocol (KF) 01–216/93 in the laboratory of O.W.P.

**Image Acquisition, Processing, and Data Analysis.** Samples were analyzed by using a Bio-Rad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 200 microscope. Fluorescence specificity was verified by sequential fluorophore excitation. NuMA foci were analyzed by using IMAGE SPACE-3D analysis program (Molecular Probes) and normalized to 3D rBM cluster-cell number by highlighting and counting each nucleus using IMAGE SPACE-MEASURE 2D. The voxel threshold was set at 0.2 μm.

**Immunoblot Analysis.** Total cell extracts (2% SDS in phosphate-buffered saline, pH 7.4, containing 1 mM Pefabloc/10 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml trypsin inhibitor type II/250 μM NaF) were prepared *in situ* for 2D cultures or from acini isolated from 3D cultures by dispase treatment (5,000 units per ml caseinolytic activity, Collaborative Research). Equal amounts of protein were separated and immunoblotted as described (18).

***In Situ* NM Preparation.** *In situ* NM preparation was as previously described (20), except that 0.05% Triton X-100 and micrococcal nuclease (5 units per ml; Sigma) were used.

**Antibody-Mediated Perturbation of Nuclear Organization.** rBM-induced acini (day 10) were permeabilized for less than 2 min *in situ* (0.01% digitonin in 25 mM Hepes, pH 7.2/78 mM potassium acetate/3 mM magnesium acetate/1 mM EGTA/300 mM sucrose/1% RIA-grade BSA), rinsed twice in digitonin-free buffer, and incubated in medium containing dialyzed specific or mock mAbs (15 μg/ml) for 48 hr, after which the cells were incubated with fresh medium for an additional 48 hr. Antibody concentrations and incubation times were determined empirically. Trypan blue dye-exclusion tests and apoptosis studies verified the absence of digitonin toxicity.

## RESULTS

**Internal Nuclear Organization Is Remodeled When HMECs Are Cultured Within a Basement Membrane.** HMT-3522 HMECs, like primary HMECs, undergo morphogenesis to form tissue-like acini when cultured in a 3D rBM (12, 18). Neither cell type undergoes acinar differentiation when cultured as 2D monolayers. In proliferating 2D cultures, NuMA was diffusely distributed in the nucleus (Fig. 1*b*) except when localized to the spindle poles in mitotic cells (15), and splicing factor SRm160 was distributed into numerous speckles of heterogenous sizes (Fig. 1*c*; ref. 16). In rBM-induced acini, NuMA was redistributed into an average of eight nuclear foci (ranging from 1 to 1.6 μm in diameter) surrounded by diffusely localized NuMA protein (Fig. 1*e*), and SRm160 was distributed into an average of seven large speckles (Fig. 1*f*). In contrast, lamin B maintained a peripheral ring-like distribution around the nucleus, with some internal localization, regardless of culture conditions (Fig. 1 *a* and *d*). The distribution pattern of these proteins was conserved in NM preparations *in situ*, where chromatin was removed before immunolocalization (staining is shown for 3D rBM cultures only (Fig. 1 *g–i*).

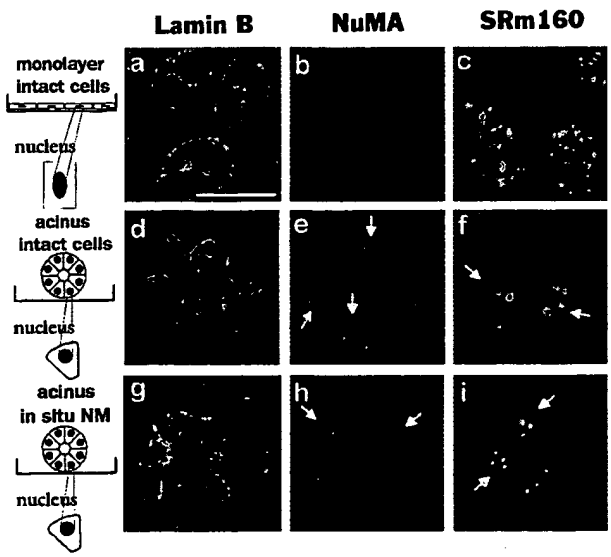



FIG. 1. NM protein redistribution in HMECs after 3D rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2-μm optical sections) of lamin B, NuMA, and splicing factor SRm160 in cells grown as 2D monolayers (*a–c*) and within 3D rBMs (*d–i*). NuMA was diffusely distributed in the nuclei of cells grown as monolayers (*b*), but reorganized into large nuclear foci in cells induced to undergo morphogenesis (acini formation) in response to a rBM (*e*). SRm160 was distributed as multiple nuclear speckles in cells cultured as monolayer (*c*), whereas it was concentrated into fewer and larger speckles in the acini (*f*). Lamin B, in contrast, consistently localized to the nuclear periphery and within intranuclear patches (*a* and *d*). The distribution of lamin B (*g*), NuMA (*h*), and SRm160 (*i*) after *in situ* NM preparation of cells cultured in 3D rBM was similar to that observed in intact cells (*d–f*). Arrows indicate nuclei found within the plane of the section. (Bar = 10 μm.)

We next examined NuMA and SRm160 distribution at different stages of 3D rBM-induced morphogenesis. After embedment in rBM, cells proliferated to form small clusters by days 3–5 but lacked β-catenin at cell–cell junctions, and collagen IV staining was discontinuous (Fig. 2 *Aa–Ac*). After growth arrest (days 6–10), cells assembled a continuous endogenous BM and formed polarized acinus-like structures with organized adherens junctions (Fig. 2 *Ad–Af*). NuMA was uniformly distributed in the nuclei of proliferating cells (Fig. 2*Ba*), but became concentrated into distinct foci of differing sizes after growth arrest (day 7; Fig. 2*Bb*), and into larger and fewer foci on completion of morphogenesis (day 10, Fig. 2*Bc*). NuMA and the splicing factor SRm160 were not colocalized in proliferating cells (Fig. 2 *Ba'* and *Ba''*), but NuMA foci and SRm160 speckles were closer together after growth arrest (Fig. 2 *Bb'* and *Bb''*) and were completely colocalized in large assemblies after the completion of morphogenesis (Fig. 2*B*, c' and c''). These spatial changes in NuMA arrangement occurred without significant modifications in the level of NuMA expression or molecular weight, as determined by using Western blot analysis (Fig. 2*Be*). These experiments demonstrate that specific NM proteins undergo spatial rearrangement during rBM-induced acinar morphogenesis. Because the existence of NuMA in differentiated tissue has been questioned (21), we studied NuMA in the normal resting human mammary gland. Intense staining was observed in the epithelial cells of acini and ducts, where NuMA was distributed in foci of different sizes and resembled the acinar stages recapitulated in 3D rBM cultures (Fig. 2*Bd*).

**Growth Arrest Is Associated With Changes in NuMA and Rb Distribution.** ECM-directed growth arrest is an early and critical step in mammary epithelial cell morphogenesis (12). To distinguish between the effect of ECM-directed growth arrest

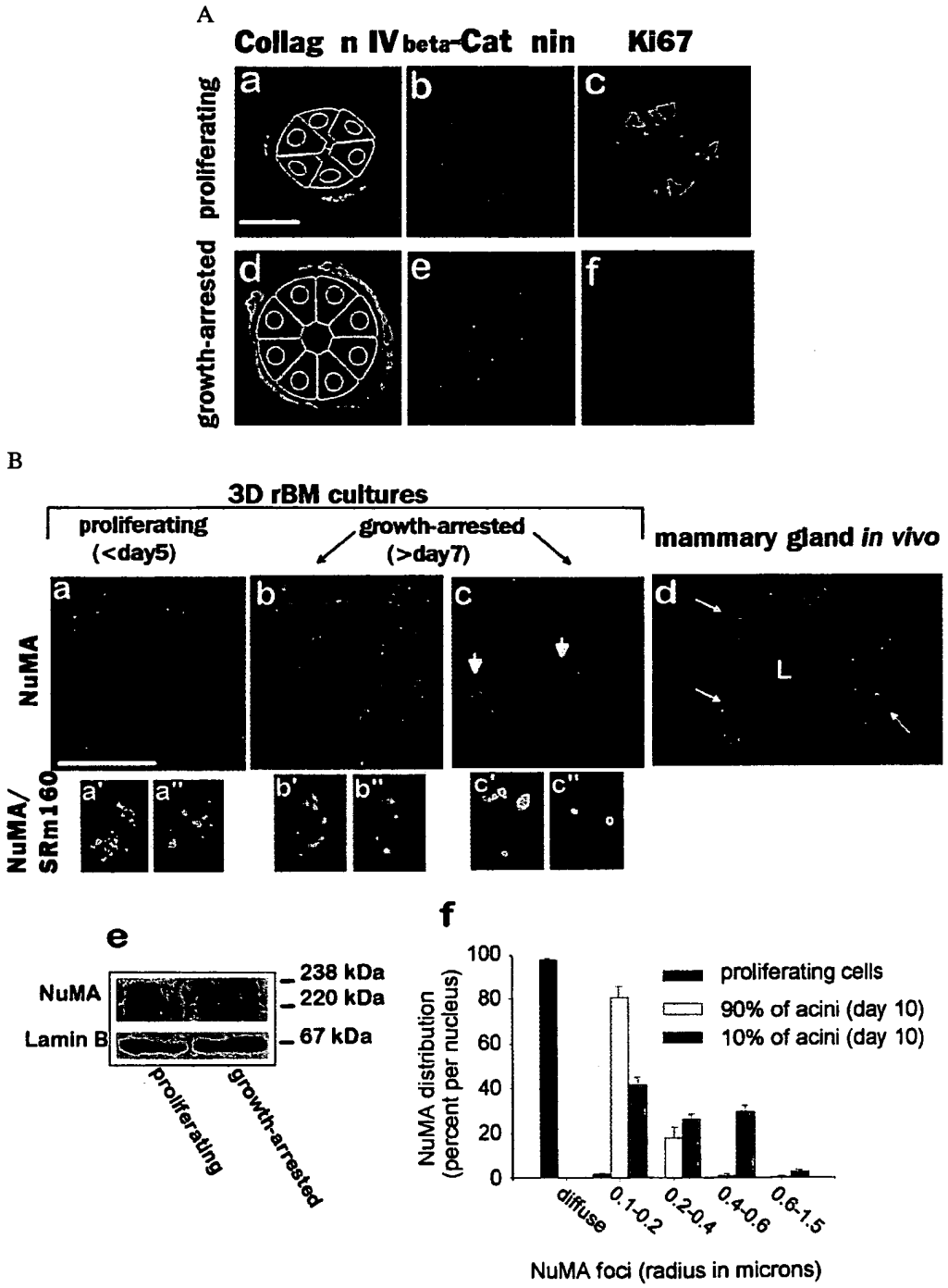



FIG. 2. (*A*) Distribution of structural proteins during rBM-induced acinar morphogenesis. Confocal fluorescence images (0.2-μm optical sections) of collagen IV, β-catenin, and Ki-67 in HMECs embedded within a rBM for 3–4 days (proliferating cells; *a–c*), and for 7–10 days (growth-arrested acini; *d–f*). Coincident with growth arrest and acinar morphogenesis, HMECs deposited an organized endogenous collagen IV-rich BM (*a* vs. *d*), whereas β-catenin relocalized from the cytosol and basal plasma membrane to sites of cell–cell adhesion (*b* vs. *e*). Acinar morphogenesis was associated with cell cycle exit, as indicated by the loss of Ki-67 staining (*c* vs. *f*). (*B*). Spatial analysis of NuMA and splicing factor SRm160 redistribution during rBM-induced acinar morphogenesis. Confocal Texas red fluorescence images (0.2-μm optical sections) of NuMA (*a–c*) and double-labeled NuMA (Texas red), and fluorescein isothiocyanate (FITC) green-stained SRm160 (*a'*, *a''*, *b'*, *b''*, *c'*, and *c''*) in HMT-3522 cells proliferating (a, a', and a'') and undergoing morphogenesis (*b*, *b'*, *b''*, *c*, *c'*, and *c''*) in response to a rBM. In proliferating cells, NuMA was diffusely distributed (*a*) and did not colocalize with SRm160 (*a'* and *a''*). After growth arrest, NuMA coalesced into foci of increasing size (0.2–2 μm; *f*) in association with the establishment of mature tissue-like structures (acini; *b* and *c*) Nine nuclei are shown in *b*. Only the larger NuMA foci observed in late morphogenesis fully colocalized with SRm160 (*b'*, *b''*, *c'*, and *c''*). (*d*) In the ductal and acinar HMECs of the mammary gland, *in vivo*, NuMA was localized in foci with a size distribution comparable to that observed in most of the HMEC nuclei of differentiating rBM cultures shown in *b*. (*e*) Western blot analysis of NuMA and Lamin B showed no difference in protein expression or size between proliferating and growth-arrested HMECs grown within rBMs. Arrows indicate nuclei. (Bars = 10 μm.)

and changes caused by tissue structure and polarity, the localization of NuMA and SRm160 was compared between growth-arrested and proliferating cells cultured in monolayers. Less than 5% of the cells remained in the cell cycle after

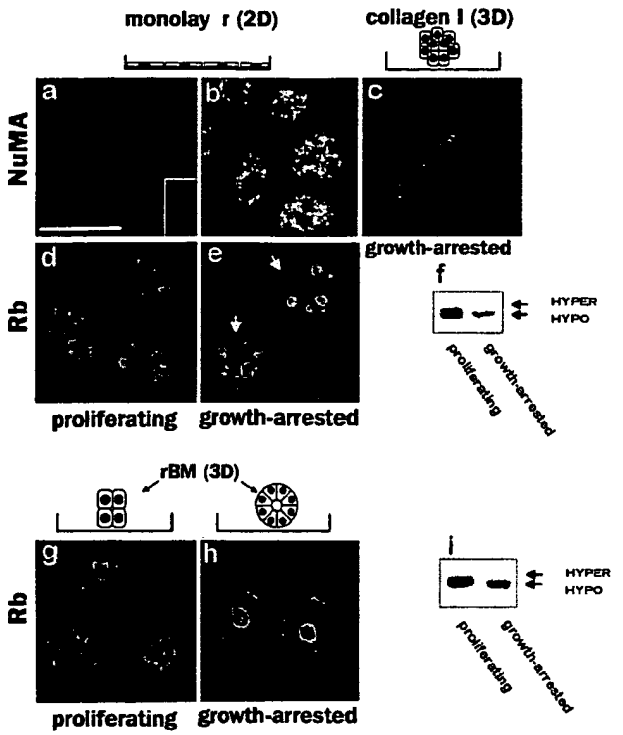



FIG. 3. Effect of growth status on the distribution of NM proteins. Confocal fluorescence images (0.2-μm optical sections) of Texas red-stained NuMA (*a–c*) and fluorescein isothiocyanate (FITC) green-stained Rb (*d*, *e*, *g*, and *h*) in cells proliferating as 2D monolayers (*a* and *d*) and within 3D rBMs (*g*) and cells growth-arrested in monolayer (*b* and *e*) and within collagen-I (*c*) or a rBM (*h*). NuMA was diffusely distributed in the nucleus of proliferating HMECs grown as monolayers (*a*) and reorganized into random aggregates on growth arrest induced by EGF removal (*b*). The settings for image recording were the same as for *a*. Aggregates appear white because of saturation of the signal. NuMA was distributed in random aggregates or in small foci in growth-arrested and BM-free cell colonies obtained after 10 days of culture within collagen-I (*c*). Rb was diffusely distributed in the nucleus of proliferating cells grown either in monolayer (*d*) or in 3D rBM (*g*); however, on growth arrest, the protein redistributed into several foci in the monolayer propagated cells (*e*) but coalesced into a central, single nuclear focus in the rBM-induced acini (*h*); the dotted line indicates outer nuclear limit. Western blot analysis of Rb in proliferating and growth-arrested cells grown as monolayers (*f*) or within a 3D rBM (*i*) shows that the hyperphosphorylated isoform was present only in proliferating cells. Arrows indicate nuclei. (Bar = 10 μm.)

growth arrest induced by EGF removal, as indicated by the absence of detectable Ki-67 immunostaining (data not shown). NuMA was uniformly distributed in the nuclei of proliferating cells but coalesced into denser areas on growth arrest (Fig. 3 *a* and *b*). The irregular geometric quality of these dense areas was distinct from the circular foci pattern observed in growth-arrested 3D rBM-grown cells. In contrast, no significant change in the multispeckled distribution of SRm160 was detected under these conditions (data not shown). The relationship between nuclear organization and growth status was further investigated by examining the distribution of the cell cycle regulator Rb. Rb redistributed from a diffuse nuclear pattern in proliferating HMECs into a few large foci in growth-arrested cells (Fig. 3 *d* and *e*). Strikingly, the distribution of Rb in the growth-arrested 2D cultures was distinct from that observed in the growth-arrested 3D cultures (compare Fig. 3 *e* and *h*), which may reflect differences in the state of growth arrest between 2D monolayer and 3D rBM cultures. The monofocal pattern of Rb observed in 3D culture coincided with growth arrest. Western blot analysis showed that hypophosphorylated Rb was associated with the NM in 3D cultures (data not shown) as was previously reported for 2D cultures (5). Moreover, the diffuse distribution observed in proliferating cells was associated with the hyperphosphorylated form of the protein (Fig. 3 *f* and *i*).

Because growth arrest in 3D rBM precedes the final stages of acinar morphogenesis (12), we examined the relationship between the large NuMA foci and the formation of a polarized endogenous BM. HMECs cultured in a 3D collagen-I matrix form growth-arrested organized colonies but do not assemble a polarized, endogenous BM (22). Therefore, we compared NuMA distribution in cells grown in rBM to those grown in type I collagen. After 12 days in collagen I, NuMA was distributed as small foci or irregular dense aggregates (Fig. 3*c*), similar to the pattern observed in growth-arrested cells in 2D cultures. Thus, NuMA redistribution into dense areas and small foci is induced by growth arrest, but the coalescence of the foci into larger and distinct structures requires the presence of a BM.

**Cross-Modulation Between NuMA Distribution, Chromatin Structure, and the Acinar Phenotype.** The degree of histone acetylation has been shown to regulate chromatin structure and gene expression (19, 23). Histone acetylation was altered in the acini by using the histone deacetylase inhibitor trichostatin A. After 2 hr of treatment, NuMA foci began to disperse, and several cells entered the cell cycle, as measured by an increase in the Ki-67 labeling index. After 24 hr of treatment, NuMA was diffusely distributed in all nuclei (Fig. 4 *e* vs. *a*), and the acinar phenotype was altered as shown by loss of the endogenous BM (Fig. 4 *f* vs. *b*), redistribution of β-catenin (Fig. 4 *g* vs. *c*), and the presence of mitotic cells, as shown by mitotic spindle-pole staining of NuMA (Fig. 4*e*, arrow). In contrast, trichostatin A did not alter the cell phenotype or the distribution of NuMA (data not shown).

Because NuMA is essential for postmitotic nuclear assembly and participates in the loss of nuclear integrity during apoptosis (24, 25), we asked whether disruption of NuMA foci in the acini could globally influence nuclear organization and affect the acinar phenotype. Rapid and reversible digitonin permeabilization was used to load cells with either anti-NuMA mAbs or with an $IgG_1$ mock mAb. The NuMA mAb B1C11, but not an N-terminal-specific mAb (clone 22; data not shown), disrupted NuMA organization, causing the protein to become diffusely redistributed within the nucleus as revealed by the secondary Ab (Fig. 4*i*). Chromatin structure was altered, as shown by the rearrangement of acetylated histone H4 distribution (Fig. 4 *l* vs. *d*). More dramatically, disruption of NuMA organization altered the acinar phenotype, as indicated by loss of the endogenously deposited BM (Fig. 4*j*). Because the loss could be prevented by treatment with GM6001, a potent metalloprotease inhibitor (Fig. 4*n*; ref. 26), we conclude that NuMA disruption led to induction and/or activation of a metalloprotease. Similar treatment of the acini with mAbs against lamins A/C or lamin B did not induce any change in histone H4 acetylation, BM integrity, or lamin distribution, even though these Abs reached their nuclear targets, as shown by secondary Ab staining (Fig. 4*n* and data not shown).

## DISCUSSION

By modifying the cellular microenvironment, we have demonstrated that nuclear organization rearranges dramatically in HMECs after growth arrest and tissue-like acinar morphogenesis (Scheme 1). The use of the 3D-rBM culture assay has enabled us also to show that alterations of nuclear organization can modify the cellular and tissue phenotype.

Previously documented changes in nuclear organization have been broadly descriptive. By systematically analyzing the distribution of three NM proteins in 2D and 3D cultures, we have determined that precise nuclear rearrangements occur

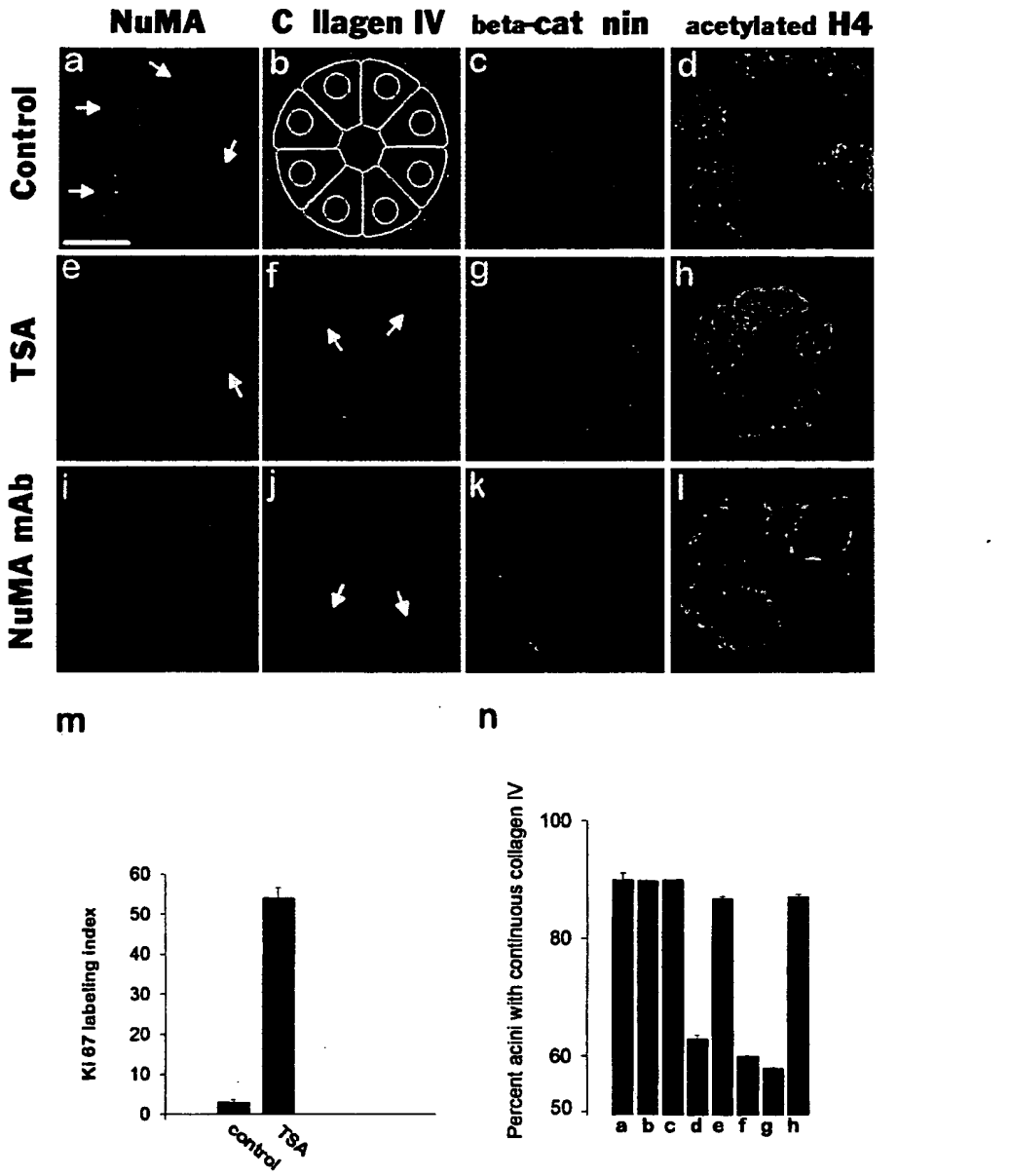
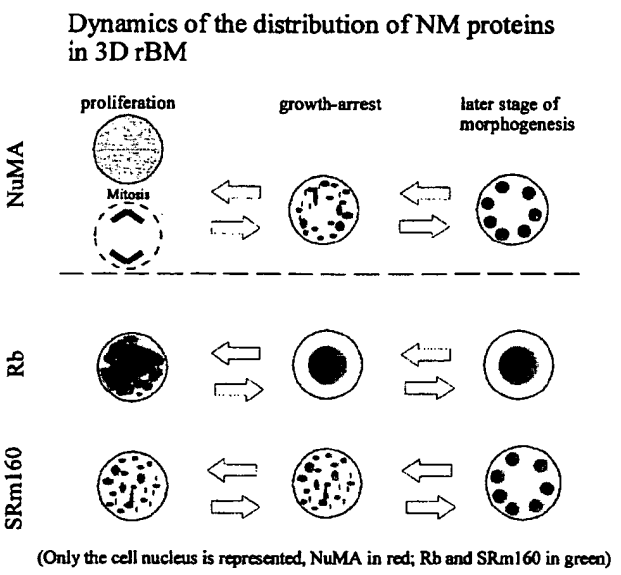



FIG. 4. Cross-modulation between chromatin structure, NM organization, and the acinar phenotype. Confocal fluorescence images (0.2-$\mu$m optical sections) of NuMA (*a*, *e*, and *i*), collagen IV (*b*, *f*, and *j*), $\beta$-catenin (*c*, *g*, and *k*), and acetylated histone H4 (*d*, *h*, and *l*) in control, trichostatin A (TSA)-treated, and NuMA mAb-incubated acini (day 10 of 3D rBM culture). (*a–d*) Nuclear organization and acinar phenotype in controls. Acini exhibit NuMA foci (*a*), an organized endogenous collagen IV-rich BM (*b*), cell–cell localized $\beta$-catenin (*c*), and dispersed acetylated H4 histone (*d*). (*e–h*) Effects of TSA on nuclear architecture and acinar phenotype. After 24 hr of TSA treatment (40 nM), >55% of the cells entered the cell cycle, as indicated by an increase in Ki-67 labeling index (*m*) and the appearance of mitotic cells (arrow in *e*). NuMA was uniformly distributed in the nuclei (*e*), collagen IV disappeared (*f*), $\beta$-catenin was released from the cell–cell interface (*g*), and the pattern of histone H4 acetylation was altered (*h*). (*i–l*) Effects of mAb-induced NuMA foci disruption on nuclear organization and acinar phenotype. Introduction of a NuMA mAb into the nuclei of the acini by using reversible digitonin permeabilization led to the disruption of NuMA foci (*i*), degradation of the collagen IV-rich BM (arrows in *j*), and the nuclear marginalization of acetylated H4 histone (*l*). There was no consistent alteration observed for $\beta$-catenin other than increased basal labeling (*k*). These effects were not observed with mock IgGs or mAbs to lamins A/C or B. (*n*) BM degradation after mAb-induced NuMA disruption in acini. Analysis of the percentage of acini with intact collagen IV-rich BMs in relation to control/digitonin-permeabilized (DP) acini (*a*), mock-IgG mAb-treated/DP acini (*b*), NuMA mAb-treated/nonpermeabilized acini (*c*), NuMA mAb-treated/DP acini (*d*), NuMA mAb-treated/DP acini + the metalloproteinase inhibitor GM6001 (*e*), NuMA mAb-treated/DP acini + the inactive metalloproteinase inhibitor GM1210 (*f*), NuMA mAb-treated/DP acini + the uPA inhibitor, aprotinin (*g*), and Lamin B mAb-treated/DP acini (*h*). Acini (>35%) degraded their endogenous BMs in response to disruption of NuMA (*d*). The BM loss could be rescued by treatment with the metalloproteinase inhibitor GM6001 (*e*), but not its inactive analogue (*f*) or a uPA protease inhibitor (*g*). (Bar = 10 $\mu$m.)

with growth arrest and after rBM-induced morphogenesis. In 3D rBM cultures, both NuMA and Rb were diffusely distributed in the nucleus of proliferating cells. After growth arrest, NuMA was relocalized into discrete foci, whereas Rb redistributed into a central nuclear mass. These patterns of distribution were different from those observed in growth-arrested cells in monolayer 2D cultures, suggesting that there may be different states of growth arrest in 2D and 3D rBM cultures (27). Because NuMA distribution in 3D collagen I cultures was comparable to that observed in growth-arrested 2D cultures, our results suggest that 3D organization of cells *per se* cannot explain the differences seen between monolayer and 3D rBM




SCHEME 1

cultures. This finding implies that BM signaling is necessary for the ultimate nuclear organization within the acini. Indeed, the presence of large and distinct NuMA foci was observed only in mature 3D rBM cultures and in adult resting mammary gland *in vivo,* where the acini were surrounded by a continuous endogenous BM. The mammary gland undergoes developmental cycles of growth and differentiation even in adults; this may account for the heterogeneity of foci size observed *in vivo* and may further explain the absence of the very large NuMA foci in subpopulations of differentiated acini (Fig. 2*Bc*). Whether the pattern of NuMA distribution indeed corresponds to different levels of differentiation *in vivo* requires further analysis.

The antibody-directed disruption of NuMA foci in the acini induced changes in the distribution pattern of acetylated histone H4, the activation of metalloprotease(s), and the loss of BM integrity. These results, as well as our observation that NuMA progressively coalesces and eventually colocalizes with enlarged splicing-factor speckles during acini differentiation, suggests that some nuclear proteins may contain the molecular information necessary for the development and/or maintenance of the acinar phenotype. Interestingly, trichostatin-induced alteration of histone acetylation in acini also led to the disruption of NuMA foci and was associated with the loss of BM and the induction of cell proliferation. Although we do not know the molecular mechanisms responsible for phenotypic alterations induced by nuclear reorganization, our experiments demonstrate also the existence of reciprocal interactions between nuclear organization, chromatin structure, and the acinar phenotype. The BM has been shown previously to be necessary for the formation and maintenance of the functional acinus (12, 28, 29). We report here that BM-induced acinar formation is associated with the distinct spatial organization of a repertoire of NM proteins and that, conversely, perturbation of nuclear organization alters the BM and influences the acinar phenotype. These results illustrate the dynamic reciprocity between the ECM and the structural organization of the nucleus, and underscore the importance of ECM–NM communication (17) in phenotypic plasticity.

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1. Berezney, R. & Coffey, D. S. (1974) *Biochem. Biophys. Res. Commun.* **60,** 1410–1417.
2. Nickerson, J. A., Blencowe, B. J. & Penman, S. (1995) *Int. Rev. Cytol.* **162A,** 67–123.
3. Nickerson, J. A., Krockmalnic, G., Van, K. M. & Penman, S. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4446–4450.
4. Odgren, P. R., Harvie, L. W. & Fey, E. G. (1996) *Proteins* **24,** 467–484.
5. Mancini, M. A., Shan, B., Nickerson, J. A., Penman, S. & Lee, W.-H. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 418–422.
6. van Wijnen, A. J., Bidwell, J. P., Fey, E. G., Penman, S., Lian, J. B., Stein, J. & Stein, G. S. (1993) *Biochemistry* **32,** 8397–8402.
7. Nardozza, T. A., Quigley, M. M. & Getzenberg, R. H. (1996) *J. Cell Biochem.* **61,** 467–477.
8. Antoniou, M., Carmo-Fonseca, M., Ferreira, J. & Lamond, A. I. (1993) *J. Cell Biol.* **123,** 1055–1068.
9. Sahlas, D. J., Milankov, K., Park, P. C. & De Boni, U. (1993) *J. Cell Sci.* **105,** 347–357.
10. Misteli, T., Caceres, J. F. & Spector, D. L. (1997) *Nature (London)* **387,** 523–527.
11. Singer, R. H & Green, M. R. (1997) *Cell* **91,** 291–294.
12. Petersen, O. W., Rønnov-Jessen L., Howlett, A. R. & Bissell, M. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9064–9068.
13. Briand, P., Petersen, O. W. & Van Deurs, B. (1987) *In Vitro Cell. Dev. Biol.* **23,** 181–188.
14. Gerace, L., Comeau, C. & Benson, M. (1984) *J. Cell Sci. Suppl.* **1,** 137–160.
15. Lydersen, B. & Pettijohn, D. (1980) *Cell* **22,** 489–499.
16. Blencowe, B. J., Issner R., Nickerson, J. A. & Sharp, P. A. (1998) *Genes Dev.* **12,** 996–1009.
17. Bissell, M. J., Hall, H. G. & Parry, G. (1982) *J. Theor. Biol.* **99,** 31–68.
18. Weaver, V. M., Petersen, O. W., Wang F., Larabell, C. A., Briand, P., Damsky, C. & Bissell, M. J. (1997) *J. Cell Biol.* **137,** 231–245.
19. Myers, C. A., Schmidhauser, C., Mellentin-Michelotti, J., Fragoso, G., Roskelley, C. D., Casperson, G., Mossi, R., Pujuguet, P., Hager G. & Bissell, M. J. (1998) *Mol. Cell. Biol.* **18,** 2184–2195.
20. He, D., Nickerson, J. A. & Penman, S. (1990) *J. Cell Biol.* **110,** 569–580.
21. Merdes, A. & Cleveland, D. W. (1998) *J. Cell Sci.* **111,** 71–79.
22. Howlett, A. R., Bailey, N., Damsky, C., Petersen, O. W. & Bissell, M. J. (1995) *J. Cell Sci.* **108,** 1945–1957.
23. Pazin, M. J. & Kadonaga, J. T. (1997) *Cell* **89,** 325–328.
24. Compton, D. A. & Cleveland, D. W. (1994) *Curr. Opin. Cell Biol.* **6,** 343–346.
25. Weaver, V. M., Carson, C. E., Walker, P. R., Chaly, N., Lach, B., Raymond, Y., Brown, D. L. & Sikorska, M. (1996) *J. Cell Sci.* **109,** 45–56.
26. Grobelny, D., Poncz, L. & Galardy, R. E. (1992) *Biochemistry* **31,** 7152–7154.
27. Dhawan, J. & Farmer, S. R. (1990) *J. Biol. Chem.* **266,** 8470–8475.
28. Streuli, C. H., Bailey, N. & Bissell, M. J. (1991) *J. Cell Biol.* **115,** 1383–1395.
29. Boudreau, N., Sympson, C. J., Werb, Z. & Bissell, M. J. (1995) *Science* **267,** 891–893.

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# A Novel Pathway for Mammary Epithelial Cell Invasion Induced by the Helix-Loop-Helix Protein Id-1

PIERRE-YVES DESPREZ,[1,2]* CLAUDIA QIAO LIN,[1,2] NICOLE THOMASSET,[1]†
CAROLYN J. SYMPSON,[1]‡ MINA J. BISSELL,[1] AND JUDITH CAMPISI[1]

*Department of Cell and Molecular Biology, Life Sciences Division, Berkeley National Laboratory, University of California, Berkeley, California 94720,[1] and Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, California 94115[2]*

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**Mammary epithelial cells undergo changes in growth, invasion, and differentiation throughout much of adulthood, and most strikingly during pregnancy, lactation, and involution. Although the pathways of milk protein expression are being elucidated, little is known, at a molecular level, about control of mammary epithelial cell phenotypes during normal tissue morphogenesis and evolution of aggressive breast cancer. We developed a murine mammary epithelial cell line, SCp2, that arrests growth and functionally differentiates in response to a basement membrane and lactogenic hormones. In these cells, expression of Id-1, an inhibitor of basic helix-loop-helix transcription factors, declines prior to differentiation, and constitutive Id-1 expression blocks differentiation. Here, we show that SCp2 cells that constitutively express Id-1 slowly invade the basement membrane but remain anchorage dependent for growth and do not form tumors in nude mice. Cells expressing Id-1 secreted a ~120-kDa gelatinase. From inhibitor studies, this gelatinase appeared to be a metalloproteinase, and it was the only metalloproteinase detectable in conditioned medium from these cells. A nontoxic inhibitor diminished the activity of this metalloproteinase in vitro and repressed the invasive phenotype of Id-1-expressing cells in culture. The implications of these findings for normal mammary-gland development and human breast cancer were investigated. A gelatinase of ~120 kDa was expressed by the mammary gland during involution, a time when Id-1 expression is high and there is extensive tissue remodeling. Moreover, high levels of Id-1 expression and the activity of a ~120-kDa gelatinase correlated with a less-differentiated and more-aggressive phenotype in human breast cancer cells. We suggest that Id-1 controls invasion by normal and neoplastic mammary epithelial cells, primarily through induction of a ~120-kDa gelatinase. This Id-1-regulated invasive phenotype could contribute to involution of the mammary gland and possibly to the development of invasive breast cancer.**

The epithelial cells of the mammary gland undergo coordinate changes in growth, differentiation, and invasion of the surrounding ECM during embryonic development and puberty, and throughout much of adulthood during each menstrual cycle. Particularly striking changes occur during pregnancy, lactation, and involution. The molecular mechanisms that control the growth and functional differentiation of mammary epithelial cells are slowly being elucidated, but far less is known about the transient invasive behavior of normal breast epithelial cells.

Normal breast epithelial cells proliferate and invade the surrounding ECM during the fetal and postnatal development of the gland, and then more vigorously at puberty as the branches of the mammary epithelial tree are formed. After puberty, there are minor waves of mammary epithelial-cell proliferation during each estrous cycle (16, 46). The most striking activity of mammary epithelial-cell proliferation and invasion occurs during pregnancy, as the gland expands in preparation for lactation (45). The proliferation and invasion of breast epithelial cells cease during late pregnancy, whereupon the cells functionally differentiate—that is, they express and secrete milk proteins (44). The epithelial cells remain proliferatively quiescent and functionally differentiated throughout lactation. At the end of lactation, the mammary gland undergoes involution, during which time there is an early and transient reactivation of epithelial-cell proliferation, followed by extensive ECM degradation and epithelial-cell death by apoptosis. The extensive remodeling of the mammary gland that occurs during involution entails the stepwise activation of several MMPs by the stromal and epithelial cells of the gland (29, 41). The involuting gland eventually returns to its prepregnancy structure.

Invasion of the ECM by normal epithelial cells must be tightly regulated and self-limiting. This control is clearly important for the mammary gland to develop and function normally. Control over normal invasive properties is also important in order to prevent neoplastic cells from invading the surrounding ECM. Most cancers develop from epithelial cells, and a hallmark of malignancy is invasion of the ECM by neoplastic epithelial cells (38). In many experimental models of tumorigenesis, an invasive phenotype develops subsequent to neoplasia and often entails expression of ECM-degrading enzymes commonly expressed by mesenchymal or stromal cells. These enzymes include the MMPs stromelysin and the 72- and 92-kDa collagenases (19, 48). It is not clear whether tumor cells express these MMPs because they are normally expressed

* Corresponding author. Mailing address: Geraldine Brush Cancer Research Institute, Stern Building, 2330 Clay St., San Francisco, CA 94115. Phone: (415) 561-1760. Fax: (415) 561-1390. E-mail: pdesprez@cooper.cpmc.org.

† Present address: INSERM Unite 433, Faculte de Medecine Laennec, 69372 Lyon, France.

‡ Present address: Searle Research and Development, Monsanto Company, Immunology Department-AA4G, North Chesterfield, MO 63198.

when epithelial cells transiently invade the ECM during normal tissue morphogenesis or because they frequently acquire mesenchymal characteristics upon transformation. It was recently shown by in situ hybridization that these MMPs are expressed by stromal fibroblasts during certain stages of ductal and alveolar mammary morphogenesis as well as during involution (29, 49).

In order to study normal and abnormal mammary epithelial-cell phenotypes, we developed a murine mammary epithelial-cell line, SCp2, whose growth and differentiation can be controlled in culture (8). SCp2 cells are an immortal line that originated from a heterogeneous cell population derived from a midpregnancy mouse mammary gland (7, 37). SCp2 cells grow well in serum on tissue culture plastic, where they express keratins and exhibit other epithelial characteristics. When serum is removed and they are given lactogenic hormones (insulin, prolactin, and hydrocortisone) and basement membrane components, SCp2 cells first arrest growth, then aggregate and form alveolar structures, and finally express high levels of several milk proteins (8, 36).

We have shown that the differentiation of SCp2 cells requires a sharp decline in the expression of the HLH protein Id-1 (9). Id genes encode a small family of proteins that prevent bHLH transcription factors from binding DNA (4). bHLH transcription factors comprise a large family of sequence-specific DNA binding proteins that activate the transcription of cell- and tissue-specific genes. bHLH proteins act as obligate dimers: they dimerize through the HLH domains and bind DNA through the composite basic domain. Id proteins contain HLH domains and therefore dimerize with bHLH proteins. However, because Id proteins lack basic domains, Id-bHLH heterodimers cannot bind DNA. Thus, Id proteins negatively regulate bHLH transcription factors. The bHLH superfamily contains both ubiquitous and lineage-specific transcription factors that direct many developmental and differentiation processes (20). Two of the four known Id proteins (Id-1 and Id-3) are nearly ubiquitously expressed, whereas the other two Id proteins (Id-2 and Id-4) have a more restricted pattern of expression (35). Thus, lineage-specific differentiation is determined by tissue-specific bHLH genes, which, in turn, are posttranslationally regulated by a small number of Id genes. Whether and how bHLH proteins participate in the differentiation of breast epithelial cells is not yet known.

Id-1 was the first Id protein to be identified (4). Since its initial discovery in myoblasts, it has been shown to be expressed by a variety of cell types and to inhibit the differentiation of myoblasts (18), several hematopoietic cell types (23, 26, 40), trophoblasts (6), and mammary epithelial cells (9). Id-1 was also found to be serum inducible in fibroblasts, where its expression is essential for progression into the S phase of the cell cycle (14). In contrast to the closely related Id-2 protein, Id-1 does not physically associate with the retinoblastoma tumor suppressor protein pRb (15, 17) but can functionally interact with a pRb-regulated pathway for entry into S phase (15).

Id-1 expression declines rapidly when SCp2 cells are induced to differentiate. As long as the cells remain in contact with a basement membrane and lactogenic hormones, Id-1 remains repressed and the cells do not proliferate, but they express milk proteins. By contrast, SCp2 cells that constitutively express Id-1 fail to differentiate, as judged by the expression of milk proteins, but nonetheless transiently arrest growth and form loose alveolar structures. After several days, cells that constitutively express Id-1 dissociate from each other and subsequently resume growth (9).

Here, we show that Id-1 expression confers upon SCp2 cells the ability to migrate and invade the basement membrane. However, cells that constitutively express Id-1 neither grow in soft agar nor form tumors in nude mice. Id-1 expression correlates strongly with expression of an apparently novel gelatinase of approximately 120 kDa, an MMP, which is also expressed during involution. The activity of this MMP was critical for the Id-1-regulated invasive phenotype. We also show that Id-1 expression correlates with the degree of differentiation and invasiveness of human breast cancer cells. The least-differentiated and most highly invasive cells express constitutively high levels of Id-1 and also secrete a 120-kDa gelatinase. Our results suggest that Id-1 is a regulator of the invasive phenotype of normal and neoplastic mammary epithelial cells and that it acts, at least in part, by controlling expression of a 120-kDa gelatinase. The invasive phenotype conferred by Id-1 is not a consequence of tumorigenic transformation, although it may be appropriated in a subset of aggressive breast cancers. Our data provide new insights into the control of breast epithelial-cell invasion and suggest that one or more bHLH transcription factors may repress the invasive phenotype in normal as well as neoplastic breast epithelial cells.

## MATERIALS AND METHODS

**Abbreviations.** AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; bHLH, basic helix-loop-helix; BSA, bovine serum albumin; DAPI, 4′,6′-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EHS, Englebreth Holm Swarm tumor; ECM, extracellular matrix; F12, Ham's F-12 medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HLH, helix-loop-helix; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-PCR.

**Cell culture.** SCp2 cells were grown in a 1:1 mixture of DMEM and F12 (DMEM-F12) containing 5% heat-inactivated FBS, insulin (5 μg/ml), and gentamicin (50 μg/ml) (growth medium) at 37°C in a humidified 5% $CO_2$ atmosphere, as previously described (8). To induce differentiation, cells were plated at $5 \times 10^4/cm^2$ atop basement membrane components in DMEM-F12 lacking serum but containing lactogenic hormones (insulin, 5 μg/ml; hydrocortisone, 1 μg/ml; prolactin, 3 μg/ml) (9). Unless otherwise indicated, cells were cultured for 5 days before analysis. Basement membrane ECM either was purified from EHS tumors by the method of Taub et al. (42) or was supplied as Matrigel from Collaborative Research.

SCp2 cells were transfected with the murine Id-1 cDNA driven by the mouse mammary tumor virus promoter as previously described (9). The transfected cells were initially pooled. Single cell-derived clones were subsequently derived by plating cells at limiting dilutions in 24-well plates. After 10 days, wells with visible colonies were trypsinized and replated onto 35-mm-diameter dishes. When nearly confluent, the cells were replated onto 100-mm-diameter dishes. The population was expanded by subculturing at a ratio of 1:4, and cells were used after 5 to 8 passages after the first 1:4 subculture.

The human breast cancer cell lines T47D, MCF-7, Hs578T, BT-549, MDA-MB-231, ZR75-1, and SKBR-3 were purchased from the American Type Culture Collection. The MDA-MB-436 cell line was originally purchased from the American Type Culture Collection and was given to us by R. Lupu (Berkeley National Laboratory). MDA-MB-435 cells were derived from the original cell line by selection in nude mice for the highly aggressive subpopulation (37a). Cells were passaged in DMEM containing 10% FBS and insulin (5 μg/ml; Sigma). For serum-free conditions, FBS was omitted from the medium.

**DNA synthesis and autoradiography.** Cells plated on coverslips were labeled with [$^3$H]methylthymidine (10 μCi/ml; 60 to 70 Ci/mmol) for 24 h, washed twice with PBS, then fixed for 5 min with a 1:1 (vol/vol) mixture of acetone and methanol at −20°C. Where indicated, cell nuclei were stained for 2 min with DAPI diluted 1:10,000 in PBS. The coverslips were air dried, coated with Kodak NTB2 emulsion (1:2 dilution), and exposed for 16 to 24 h. The coverslips were developed with D-19, fixed with Kodak Rapid-Fix, and viewed by phase-contrast microscopy.

**Boyden chamber invasion assays.** Invasion assays were performed in modified Boyden chambers with 8-μm-pore-size filter inserts for 24-well plates (Collaborative Research). Filters were coated with 10 to 12 μl of ice-cold basement membrane ECM at 8 to 12 mg of protein/ml. Cells ($0.5 \times 10^5$ to $1 \times 10^5$) were added to the upper chamber in 200 μl of DMEM-F12. The lower chamber was filled with 300 μl of NIH 3T3 cell-conditioned medium. Where indicated, GM6001 was added at 0.2 mM to both chambers immediately after cell plating. After a 16- to 20-h incubation, the cells were fixed with 2.5% glutaraldehyde in PBS and stained with 0.5% toluidine blue in 2% $Na_2CO_3$. Cells that remained in the basement membrane or attached to the upper side of the filter were removed

with paper towels. Cells on the lower side of the filter were examined by light microscopy and counted.

**Anchorage-dependent growth assays.** Liquefied 2% agarose was mixed with an equal volume of 2× DMEM-F12 growth medium lacking serum and supplemented with insulin (10 μg/ml) and gentamicin (100 μg/ml) (2× medium). One milliliter of the mixture was layered onto 35-mm-diameter dishes to create a 1% agarose base. Liquefied 0.6% agarose was mixed with an equal volume of 2× medium, and 10 ml of this solution was mixed with 1 ml of growth medium containing $10^5$ cells to yield $10^4$ cells/ml in 0.27% agarose; 1 ml of this cell suspension was layered on top of the 1% agarose base, and 1 ml of DMEM-F12 containing 5% FBS was added. The cells were incubated for 14 days, after which representative fields were photographed under phase-contrast microscopy.

**Tumorigenicity assays.** Cells were injected subcutaneously into nude mice at $4 \times 10^6$ cells per site, two sites per animal, and two animals for each cell type (TCL1, SCg6, SCp2, SCp2–antisense Id-1, and SCp2–Id-1). Animals injected with TCL1 and SCg6 cells developed easily detectable tumors (at least 1 $cm^3$) within 3 weeks and were sacrificed after 4 weeks. The remaining animals remained tumor negative for a minimum of 5 months.

**Immunofluorescence.** Cells cultured on coverslips were washed with PBS, fixed for 5 min with acetone-methanol (1:1, vol/vol) at −20°C, permeabilized for 5 min with 1% Triton X-100 in PBS, and washed with PBS. A rabbit polyclonal antiserum raised against bovine keratins (Dako, Carpinteria, Calif.) was diluted 1:10 in 0.2% BSA in PBS and applied for 60 min at 37°C, followed by three washes in PBS. The coverslips were then incubated with biotin-conjugated anti-rabbit antibody (1:100 dilution; Amersham Corp.) for 30 min at 37°C and were washed three times in PBS. Finally, the coverslips were incubated with fluorescein isothiocyanate-conjugated streptavidin (1:100 dilution; Amersham Corp.) for 30 min at 37°C and were washed in PBS. Cell nuclei were stained with DAPI, as described above, and the coverslips were mounted in glycerol-gelatin (Sigma) for viewing by epifluorescence.

**RNA isolation and analysis.** Total cellular RNA was isolated and purified as described by Chomczynski and Sacchi (5). The RNA (10 μg) was size fractionated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N from Amersham Corp.). The membrane was hybridized to $^{32}P$-labeled probes prepared by random oligonucleotide priming, washed, and exposed to XAR-5 film for autoradiography as described by Maniatis et al. (31). The β-casein probe was the 540-bp mouse cDNA (from J. Rosen, Baylor College of Medicine, Houston, Tex.), and the Id-1 probe was either the murine Id-1 cDNA (4) or the human Id-1 cDNA (14).

**RT-PCR and Southern analysis.** Transcripts for murine gelatinases A (72-kDa MMP) and B (92-kDa MMP) were detected by RT-PCR. cDNA was synthesized from total RNA by using SuperscriptII Reverse TranscriptaseII (Gibco-BRL), and 100 ng was used for PCR. The 5′ and 3′ PCR primers were TTGAGAAG GATGGCAAGTATGG and ACACCTTGCCATCGTTGC for gelatinase A, GGCGTGTCTGGAGATTCGA and AGGGTCCACCTTGGTCACC for gelatinase B, and ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGC TGTA for GAPDH. PCR was performed in 20 mM Tris-HCl (pH 8.8)–2 mM $MgSO_4$–10 mM KCl–10 mM $(NH_4)_2SO_4$–0.1% Triton X-100–100 μg of BSA/ml–0.125 mM deoxynucleoside triphosphates–0.8 μM each PCR primer–0.05 U of *Pfu* DNA polymerase/μl by using 35 cycles for amplification of gelatinase cDNAs and 25 cycles for amplification of GAPDH cDNA. The cycle conditions were 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 30 s of extension at 72°C. For Southern analysis, one-fifth of the PCR reaction product was separated on a 2.1% agarose gel, transferred to a nylon membrane (Hybond N+), and hybridized with cDNA inserts labeled with $^{32}P$ by random priming. cDNAs encoding murine gelatinase A or B (33, 34) were a gift from Z. Werb, University of California, San Francisco, and the GAPDH cDNA was obtained from Clontech (Palo Alto, Calif.). Hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 50% formamide at 42°C overnight. The membranes were washed at a final stringency of 0.2× SSC and 0.1% sodium dodecyl sulfate at 68°C and were exposed to XAR-5 film for autoradiography.

**Zymography.** Proliferating cells ($10^6$ in 100-mm-diameter dishes) were shifted to serum-free medium for 2 to 3 days, after which they were given 10 ml of fresh serum-free medium. Forty-eight hours later, the conditioned medium was collected and concentrated 10- to 15-fold by using 10-kDa-cutoff filters (Millipore, Bedford, Mass.). The concentrated medium was analyzed on casein and gelatin substrate gels, as described by Fisher and Werb (10) and Talhouk et al. (41). Briefly, gels consisted of 8 to 10% polyacrylamide and 3 mg of α-casein or gelatin (Sigma)/ml. Concentrated conditioned medium was mixed with nonreducing Laemmli sample buffer and incubated at 37°C for 15 min. After electrophoresis, the gels were incubated for 1 h in 2.5% Triton X-100 at room temperature, followed by 24 to 48 h in substrate buffer (100 mM Tris-HCl [pH 7.4]–15 mM $CaCl_2$) in the absence or presence of GM6001 (0.2 mM in DMSO; supplied by Glycomed Corporation and obtained from Z. Werb [12]), EDTA (10 mM), *ortho*-phenanthroline (1 mM in DMSO; Sigma), PMSF (5 mM), or AEBSF (0.5 mM; Calbiochem). Where appropriate, control gels were incubated with buffer containing solvent only. The gels were stained with Coomassie blue for 30 min and were destained with 30% methanol–10% acetic acid. Caseinase and gelatinase activities were visible as clear bands, indicative of proteolysis of the substrate protein.

## RESULTS

**Id-1 induces an invasive, migratory phenotype in mammary epithelial cells.** SCp2 mammary epithelial cells grow as a monolayer in 5% serum. When given lactogenic hormones and basement membrane ECM in serum-free medium, they arrest growth, form three-dimensional alveolar structures, and express the milk protein β-casein (8). Alveoli formed by SCp2 cells are stable, maintaining their structure and β-casein expression for more than 2 weeks. Under these conditions, Id-1 is not expressed. By contrast, SCp2 cells that constitutively express Id-1 form poorly compacted alveoli that become increasingly disorganized; after 6 to 8 days, cells at the periphery detach from the structure and synthesize DNA (9).

Using a pooled population of SCp2 cells that constitutively express a murine Id-1 transgene (SCp2–Id-1 cells) (9), we more precisely monitored the fate of cells that detached from the alveolar structure. Within 10 days, approximately 30 to 40% of the SCp2–Id-1 alveolar structures showed substantial disintegration. Following detachment from the alveolar structure, SCp2–Id-1 cells actively invaded and migrated through the surrounding ECM (Fig. 1). The migrating cells had an elongated nuclear morphology, compared to the rounded nuclei of cells in the early stages of disaggregation. Initial detachment and invasion occurred in the absence of cell proliferation (Fig. 1A). However, 2 to 4 days after initial detachment, SCp2–Id-1 cells that had migrated extensively through the ECM were abundant, and many of these cells synthesized DNA (Fig. 1B to D). For the most part, DNA synthesis was evident only in cells that had migrated some distance from the alveolar structure. Thus, the initiation of invasion and migration was not due to resumption of growth; rather, cells resumed proliferation only after they had detached and migrated from the three-dimensional structure. As previously described (9), spheres comprised of control cells transfected with the vector alone were very stable, remaining viable and morphologically unchanged even after more than 10 days on basement membrane ECM.

To quantify the invasion and migration of SCp2–Id-1 cells, they and control cells were assayed in Boyden chambers (2). Cells were added to the upper portion of the chamber; conditioned medium from mouse fibroblasts, used as a source of chemoattractants (2), was added to the lower compartment. The porous filter separating the two compartments was coated with basement membrane ECM. After a 16- to 20-h incubation, cells that had migrated through the ECM to the lower surface of the filter were fixed, stained, and counted (Fig. 2). The 16- to 20-h incubation time ensured that only a small fraction of invasive cells migrated through the filter, which in turn ensured that the fraction of migratory cells was small enough to score reliably.

Four types of cells were compared in this assay: (i) parental SCp2 cells, (ii) SCp2 cells transfected with an insertless vector, (iii) SCp2–Id-1 cells, and (iv) SCp2 cells transfected with the Id-1 cDNA in the antisense orientation. Of these cell types, only SCp2–Id-1 cells were invasive. Under these assay conditions, none of the control (parental or insertless-vector) cells and none of the cells expressing antisense Id-1 migrated through the filter. By contrast, 0.7 to 1% of a population of one of the most invasive breast cancer cell lines (MDA-MB-231, previously described [43]) migrated through the filter, although only about 0.05% of the SCg6-transformed cells, which were previously shown to be invasive (28), migrated through the ECM to the lower surface of the filter (data not shown). In the case of the SCp2–Id-1 cells, 0.2 to 0.3% migrated through the filter. Thus, SCp2–Id-1 cells, which were transfected with a single gene, were 20 to 30% as invasive as one of the most

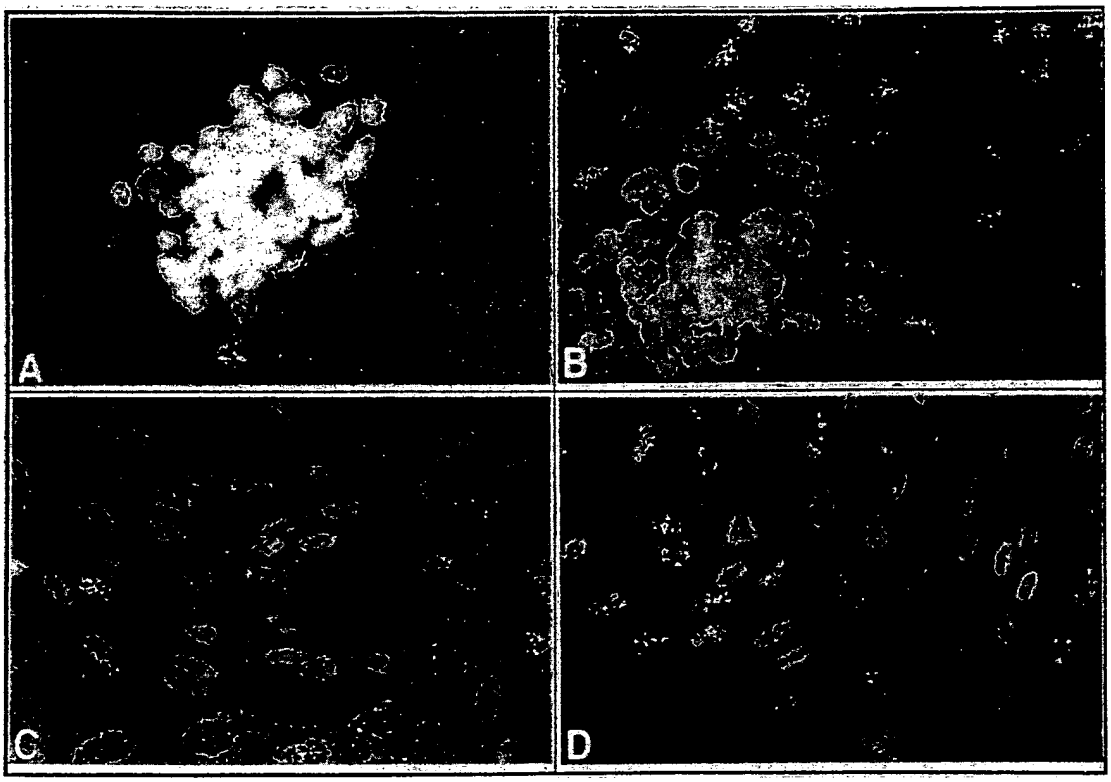


FIG. 1. Instability of the three-dimensional organization and loss of growth arrest of SCp2–Id-1 cells. A pooled population of SCp2–Id-1 cells was induced to differentiate for 8 (A), 10 (B), or 12 (C and D) days, [$^3$H]thymidine was added for 24 h preceding fixation, and the cells were then stained with DAPI and processed for autoradiography as described in Materials and Methods. Shown are the DAPI fluorescence and autoradiography. Depending on the batch of EHS ECM or Matrigel, disaggregation of the three-dimensional structures and resumption of DNA synthesis occurred 1 to 2 days earlier or later than in the experiments for which results are shown here. Magnification, ×300.

aggressive breast cancer cell lines (which harbors multiple mutations) and four- to sixfold more invasive than their SCg6-transformed counterparts.

We conclude that constitutive expression of the Id-1 gene can induce an invasive and migratory phenotype in nontransformed and nontumorigenic SCp2 mammary epithelial cells.

**Constitutive Id-1 expression is not sufficient for anchorage-independent growth or tumorigenicity.** In many model systems of malignant transformation, unregulated expression of normal or activated proto-oncogenes drives cell proliferation, and invasiveness often develops subsequent to, or concomitant with, tumorigenicity. Although Id-1 did not appear in this regard to act like a typical oncogene, we nonetheless asked whether constitutive Id-1 expression transformed SCp2 cells, using the criteria of anchorage-independent growth and tumorigenicity in nude mice.

We first tested the ability of the cells to grow in an anchorage-independent manner. As expected, control cells and cells transfected with Id-1 in the antisense orientation failed to grow in soft agar (Fig. 3A and C). Similarly, SCp2–Id-1 cells failed to form colonies in soft agar, remaining as single cells for at least 14 days (Fig. 3B). It is interesting that, in soft agar, SCp2–Id-1 cells appeared twice as large as control cells; the reason for this size increase is not known. Malignant TCL1 cells (isolated from a murine mammary tumor [28]), used as a positive control, formed large colonies after 14 days in soft agar (Fig. 3D). We conclude that constitutive expression of Id-1 does not

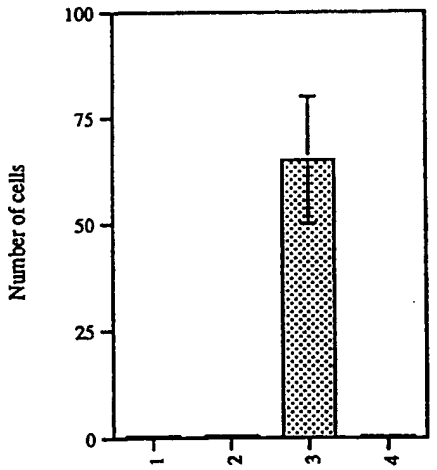


FIG. 2. SCp2–Id-1 cells invade the ECM and migrate in a Boyden chamber. Parental SCp2 cells (lane 1), SCp2 cells transfected with an insertless vector (lane 2), SCp2–Id-1 cells (lane 3), and SCp2 cells transfected with Id-1 in the antisense orientation (lane 4) were plated on ECM-coated filters in Boyden chambers; the number of cells that migrated through the filter after 16 to 20 h was determined as described in Materials and Methods. Error bars indicate standard deviations from three or four independent fields. The data shown are from one of five independent experiments which showed very similar differences among the cell types.

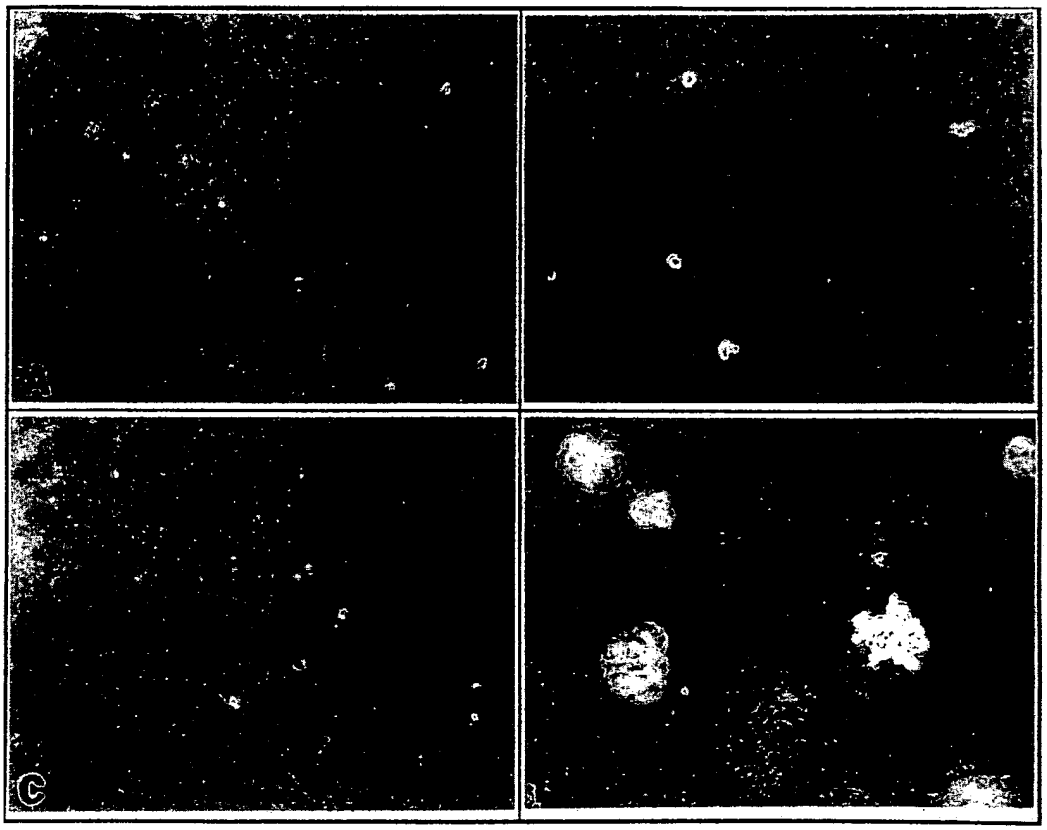

FIG. 3. SCp2–Id-1 cells do not grow in an anchorage-independent manner. Parental SCp2 cells (A), SCp2–Id-1 cells (B), SCp2 cells expressing an Id-1 antisense vector (C), and TCL1 mammary tumor cells (D) were seeded in soft agar as described in Materials and Methods and photographed 14 days later. Magnification, ×50.

induce anchorage-independent growth in SCp2 mammary epithelial cells.

We next tested SCp2–Id-1 cells for their ability to form tumors. Cells were injected subcutaneously into nude mice. The positive control, TCL1 cells, formed tumors (at least 1 $cm^3$) within 3 weeks (Table 1). The same was true for SCg6, a cell line with mesenchymal and transformed properties that was isolated from the same population from which SCp2 cells were isolated (8) (Table 1). By contrast, neither parental SCp2 cells, SCp2 cells expressing the Id-1 antisense cDNA, nor SCp2–Id-1 cells formed tumors after 5 months (Table 1).

We conclude that constitutive Id-1 expression in SCp2 mammary epithelial cells is not sufficient to lead to the transformed phenotypes of anchorage-independent growth in culture and in vivo, despite its ability to induce an invasive phenotype.

**Isolation and characterization of cloned SCp2–Id-1 cells.** The experiments described thus far used a pooled population of SCp2–Id-1 cells, which is heterogeneous with respect to Id-1 expression. To eliminate this heterogeneity and better define the role of Id-1 in inducing an invasive phenotype, we isolated single-cell-derived SCp2–Id-1 clones that expressed the Id-1 transgene to varying levels. The clones were assessed for cytokeratin filaments (a general characteristic of epithelial cells), morphology in monolayer culture, and ability to form alveolar structures in response to basement membrane ECM. In addition, RNA was isolated 5 days after the cells were exposed to basement membrane and hormones and was analyzed for Id-1 and β-casein mRNA. The Id-1 transgene mRNA was distinguishable from the endogenous Id-1 mRNA by its slightly larger size; the endogenous transcript was barely detectable under these conditions (9).

One subclone, SCp2–Id-1A cells, did not express detectable Id-1 transgene mRNA (Fig. 4a, lane 1). These cells grew as compact colonies in monolayer culture and expressed cytokeratin filaments (Fig. 4b, panel B). They also differentiated similarly to untransfected SCp2 cells, as judged by their ability to express high levels of β-casein mRNA (Fig. 4a, lane 1) and form stable alveolar structures (data not shown), like untransfected SCp2 cells. These cells were therefore used as negative controls.

SCp2–Id-1B and SCp2–Id-1C cells expressed moderate levels of the Id-1 transgene, which were below the levels of Id-1

TABLE 1. SCp2–Id-1 cells are not tumorigenic[a]

| Cell type | No. of tumors/no. of sites injected (time) |
|---|---|
| TCL1 | 4/4 (after 3 wk) |
| SCg6 | 4/4 (after 3 wk) |
| SCp2 | 0/4 (after 5 mo) |
| SCp2–antisense Id-1 | 0/4 (after 5 mo) |
| SCp2–Id-1 | 0/4 (after 5 mo) |

[a] Cells ($4 \times 10^6$ per site) were injected into two sites per animal, and two animals were used for each cell type, as described in Materials and Methods. The tumors that developed were at least 1 $cm^3$.

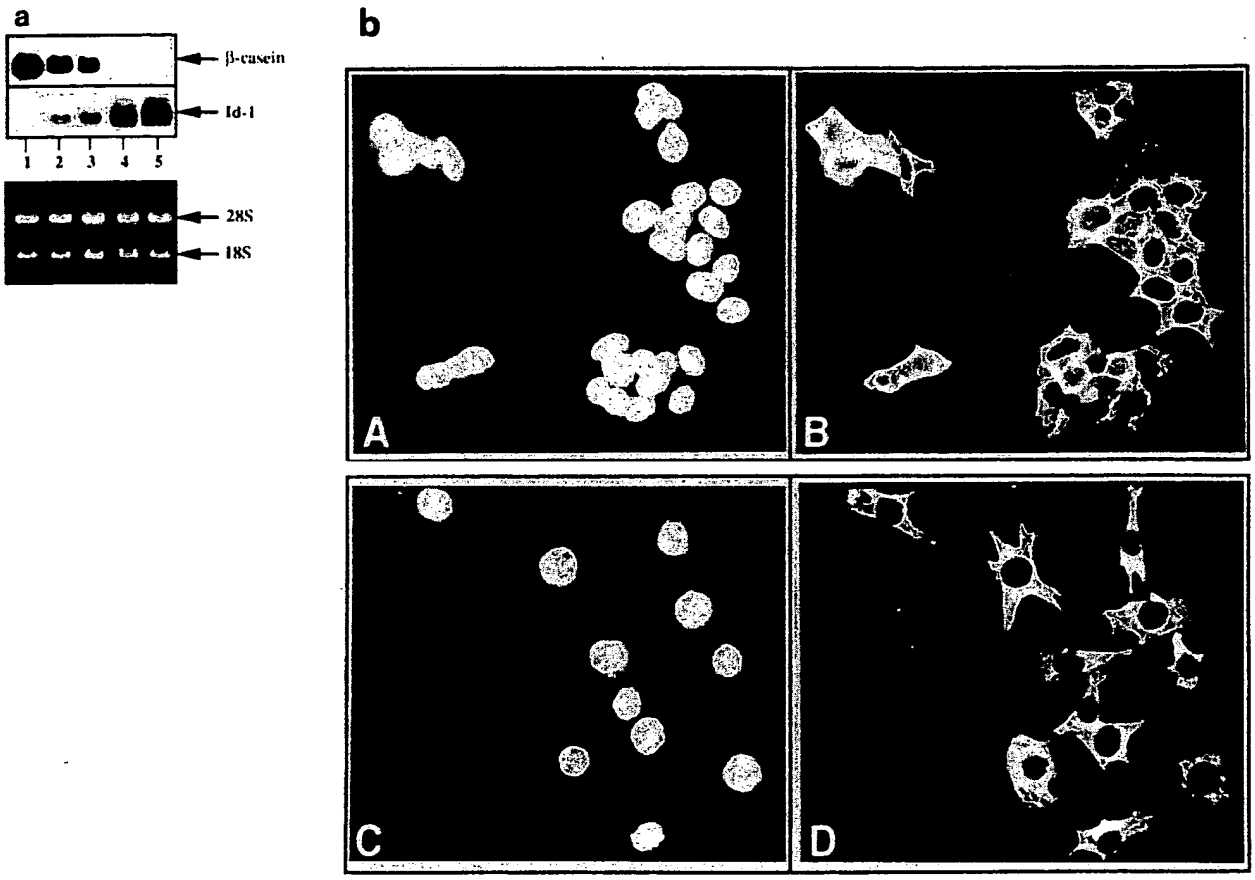


FIG. 4. Characterization of SCp2 cell clones expressing constitutive Id-1. (a) SCp2-Id-1 cells were plated at limiting dilution, and five independent clones (SCp2-Id-1A through SCp2-Id-1E) were isolated and amplified. Cells from each of these clones were exposed to basement membrane and hormones for 5 days and were analyzed for expression of the Id-1 transgene and β-casein mRNA, as described in Materials and Methods. Shown are the autoradiogram of the Northern blot and the ethidium bromide-stained Northern gel made to confirm RNA integrity and quantitation. Lane 1, SCp2-Id-1A; lane 2, SCp2-Id-1B; lane 3, SCp2-Id-1C; lane 4, SCp2-Id-1D; lane 5, SCp2-Id-1E. (b) SCp2-Id-1A (A and B) and SCp2-Id-1E (C and D) cells were grown in monolayer culture, fixed, and stained with DAPI (A and C) or processed for immunofluorescence by using a pan-keratin antibody (B and D), as described in Materials and Methods.

mRNA expressed by proliferating control cells. These cells expressed lower levels of β-casein mRNA than SCp2-Id-1A cells (Fig. 4a, lanes 2 and 3), but they expressed cytokeratin filaments and formed alveolar structures (data not shown).

Finally, SCp2-Id-1D and SCp2-Id-1E cells expressed high levels of the Id-1 transgene and undetectable levels of β-casein (Fig. 4a, lanes 4 and 5). In monolayer culture, SCp2-Id-1E cells were less cuboidal and grew as more-dispersed entities than SCp2-Id-1A cells (Fig. 4b, panels C and D). Their failure to express β-casein was not due to a loss of epithelial characteristics. SCp2-Id-1E cells, which expressed the highest levels of Id-1, as well as SCp2-Id-1D cells (data not shown), expressed cytokeratin filaments (Fig. 4b, panel D). However, SCp2-Id-1D and SCp2-Id-1E cells, like the pooled SCp2-Id-1 cells, formed only loose alveolar structures, from which they eventually detached and invaded the ECM (see Fig. 7) (data not shown).

These results confirm in cloned populations that mammary epithelial cells constitutively expressing Id-1 do not undergo a complete epithelial-to-mesenchymal transition; they retain some epithelial-cell characteristics (such as keratin expression) but fail to functionally differentiate and to maintain three-dimensional organization on the ECM. SCp2-Id-1A and SCp2-Id-1E cells, which express undetectable and high levels of the Id-1 transgene, respectively, were used for further studies.

**A potentially novel metalloproteinase is secreted by Id-1-expressing cells.** The ability of SCp2-Id-1 cells to invade the ECM suggested that Id-1 might induce expression of ECM-degrading proteases. The major classes of proteases that degrade ECM are serine, cysteine, and aspartyl proteases, and metalloproteinases (10). The $Zn^{2+}$-containing, $Ca^{2+}$-stabilized MMPs are of particular interest because they are implicated in the remodeling of the mammary gland during involution (29, 41) and the initial steps of tumor-cell invasion (25). Of the major MMPs, interstitial collagenase (56 kDa) and gelatinases A (72 kDa) and B (92 kDa) are detectable on gelatin substrate gels, whereas stromelysins (57 kDa for stromelysin-1) and matrilysin (30 kDa) are detectable on casein substrate gels (reviewed by Fisher and Werb [10]; see also reference 22).

We examined the secretion of proteases by SCp2-Id-1A and SCp2-Id-1E cells, using conditioned medium and gelatin or casein substrate gel zymography. Cells were incubated in serum-free medium for 3 days prior to collection of conditioned medium for zymography. Under these conditions, the endogenous Id-1 gene is not expressed (9), and SCp2-Id-1A and

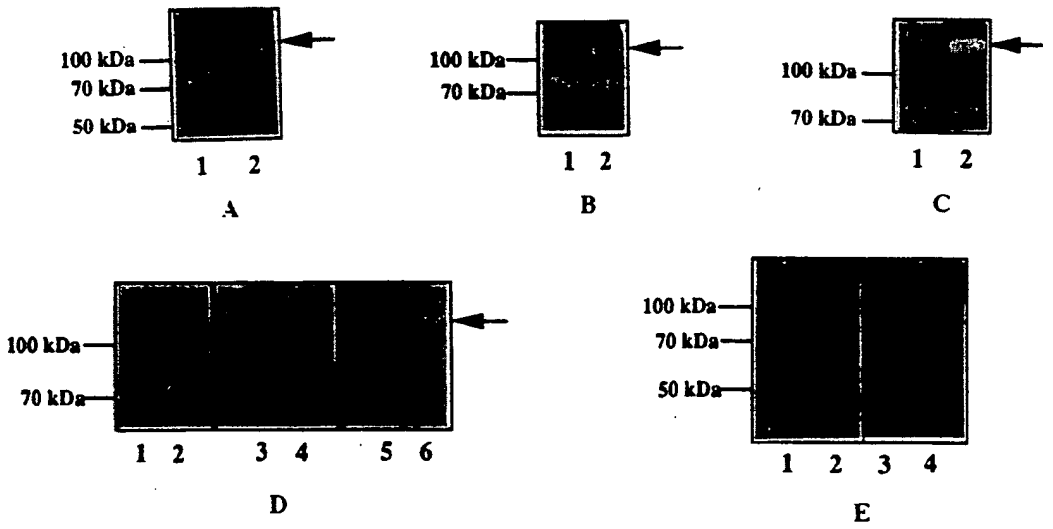


FIG. 5. Expression of a 120-kDa gelatinase by Id-1-expressing mammary epithelial cells. (A) Gelatin zymogram of conditioned media from SCp2–Id-1A (lane 1) and SCp2–Id-1E (lane 2) cells. Cells were cultured in serum-free medium, and conditioned media were harvested and analyzed on a gelatin substrate gel, as described in Materials and Methods. (B) Gelatin zymogram of conditioned media from SCp2–Id-1A cells either growth arrested by serum deprivation (lane 1) or growing in 5% serum (lane 2). (C) Gelatin zymogram of conditioned media from control SCp2 cells (lane 1) and an uncloned SCp2–Id-1-transfected pooled population (lane 2). Cells were cultured in serum-free medium. (D) Gelatin zymogram of SCp2–Id-1A (lanes 1, 3, and 5) and SCp2–Id-1E (lanes 2, 4, and 6) cell-conditioned media incubated with DMSO (lanes 1 and 2), the MMP inhibitor GM6001 (0.2 mM in DMSO) (lanes 3 and 4), or the serine proteinase inhibitor PMSF (5 mM in DMSO) (lanes 5 and 6). (E) Casein zymogram of conditioned media from SCp2–Id-1A (lanes 1 and 3) and SCp2–Id-1E (lanes 2 and 4) cells incubated with DMSO (lanes 1 and 2) or GM6001 (lanes 3 and 4). In panels A through D, arrows mark the positions of the 120-kDa MMP.

SCp2–Id-1E express undetectable and high levels of the Id-1 transgene, respectively.

Gelatin substrate gels showed that SCp2–Id-1A and SCp2–Id-1E cells differed only in the secretion of a high-molecular-mass (approximately 120-kDa) gelatinase. The 120-kDa gelatinase was abundantly expressed by serum-deprived SCp2–Id-1E cells (Fig. 5A, lane 2) as well as SCp2–Id-1D cells (data not shown). Secretion of this 120-kDa gelatinase was not due to clonal variation. Conditioned medium from the uncloned SCp2–Id-1 pooled population also showed a gelatinase of ~120 kDa (Fig. 5C, lane 2). This gelatinase was undetectable in serum-deprived SCp2–Id-1A (Fig. 5A, lane 1) and control SCp2 (Fig. 5C, lane 1) cells. Thus, secretion of a 120-kDa gelatinase correlated with Id-1 expression.

Secretion of the 120-kDa gelatinase correlated with expression of the endogenous Id-1 gene as well as with that of the Id-1 transgene. Thus, the 120-kDa proteinase was secreted by SCp2–Id-1A cells (in which expression of the Id-1 transgene is undetectable) while they were proliferating in monolayer culture (Fig. 5B, lane 2). Under these conditions, the endogenous Id-1 gene is expressed at high levels (9).

The 120-kDa gelatinase had characteristics of an MMP. It was sensitive to the MMP inhibitors GM6001 (Fig. 5D, lane 4), EDTA, and *ortho*-phenanthroline (data not shown). By contrast, it was insensitive to the serine protease inhibitors PMSF (Fig. 5D, lane 6) and AEBSF (data not shown). The 120-kDa MMP appeared to be the only MMP secreted by Id-1-expressing cells. The two gelatinases with apparent molecular sizes of 70 and 90 kDa, which were expressed by both SCp2–Id-1A and SCp2–Id-1E cells, were not inhibited by any of the MMP inhibitors GM6001 (Fig. 5D, lane 4), *ortho*-phenanthroline, and EDTA (data not shown), and therefore neither is likely to be gelatinase A or B.

Casein substrate gels showed one major caseinase of approximately 50 kDa that was expressed by both SCp2–Id-1A and SCp2–Id-1E cells. This protease was not inhibited by the metalloproteinase inhibitor GM6001 (Fig. 5E). Therefore, it is most likely not the metalloproteinase stromelysin-1.

To definitively rule out the possibility that gelatinases A and B were expressed in SCp2 cells, as well as the possibility that the 120-kDa MMP was a complex between gelatinase B and its carrier protein (21), we analyzed RNA by PCR and Southern blotting for gelatinase-A and -B mRNAs. SCp2–Id-1A cells, which do not express the Id-1 transgene, SCp2–Id-1E cells, which express high levels of the Id-1 transgene, and the mesenchyme-like mammary SCg6 cells were deprived of serum for 3 days before RNA was extracted and synthesized into cDNA for PCR analysis (Fig. 6). The 326-bp PCR product expected from the gelatinase-A cDNA and the 190-bp product expected for the gelatinase-B cDNA were detected only in SCg6 cells (Fig. 6, lane 3). We conclude that SCp2 cells, whether or not they express Id-1, do not express gelatinase A or B and that therefore the 120-kDa gelatinase is not a gelatinase B-containing complex (21).

We conclude that SCp2 mammary epithelial cells secrete a single detectable MMP, having an apparent molecular size of 120 kDa, when they express Id-1. This MMP does not belong to the stromelysin subclass of MMPs, which degrades casein, but rather is a type IV collagenase MMP family member and thus degrades gelatin, a denatured collagen.

**The Id-1-related MMP is essential for the invasive phenotype of SCp2 cells.** Because the 120-kDa MMP appears to be the only proteinase whose secretion correlates with Id-1 expression, and constitutive Id-1 expression renders cells invasive, we explored the possibility that this MMP is critical for the invasive phenotype of mammary epithelial cells.

We first tested the abilities of SCp2–Id-1A and SCp2–Id-1E cells to invade basement membrane ECM in a Boyden chamber invasion assay (Fig. 7). SCp2–Id-1A cells, like untransfected SCp2 cells (Fig. 2), were not invasive, or only minimally invasive, in this assay (Fig. 7). Under the assay conditions, the endogenous Id-1 gene is not expressed and SCp2–Id-1A cells

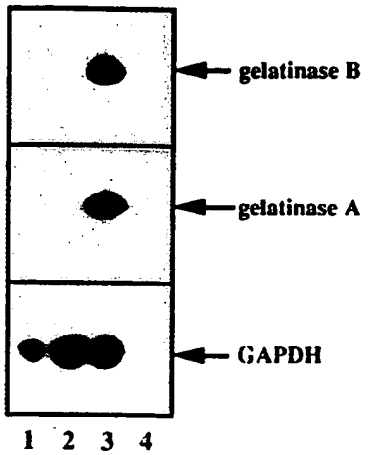


FIG. 6. SCp2 cells do not express gelatinase A or B. SCp2 cells were serum deprived for 3 days before RNA was extracted, transcribed into cDNA, and analyzed by PCR for gelatinase-A and -B cDNA sequences, as described in Materials and Methods. Arrows indicate the positions of the amplified products for gelatinases A and B and the control gene, encoding GAPDH. Lane 1, SCp2-Id-1A cells; lane 2, SCp2-Id-1E cells; lane 3, SCg6 cells; lane 4, no cDNA control.

express undetectable levels of the Id-1 transgene. By contrast, SCp2-Id-1E cells, like uncloned SCp2-Id-1 cells (Fig. 2), were demonstrably invasive under the same conditions, consistent with the high levels of the Id-1 transgene expressed by these cells.

To test the role of the 120-kDa MMP in the invasive phenotype induced by Id-1, we used MMP inhibitors in the invasion assay. We first tested the toxicities of two compounds, GM6001 and phenanthroline. SCp2 cells were treated with either compound, the solvent (DMSO), or nothing for the duration of the invasion assays (20 h), and viability was assessed by trypan blue exclusion. There were no differences in viability among untreated, DMSO-treated, and GM6001-treated cells (data not shown). By contrast, all the phenanthroline-treated cells died within 20 h of treatment. We therefore used GM6001 in the invasion assay. GM6001 reduced the invasiveness of SCp2-Id-1E cells about fourfold (Fig. 7, lane 3). Because the 120-kDa gelatinase is the only detectable MMP expressed by these cells, this result suggests that much of the invasive phenotype induced by Id-1 can be attributed to the 120-kDa MMP.

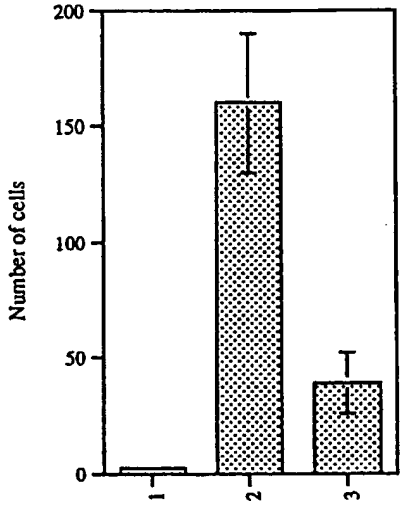


FIG. 7. The invasive phenotype of Id-1-expressing cells is repressed by an MMP inhibitor. SCp2-Id-1A cells in 0.5% DMSO (lane 1), SCp2-Id-1E cells in 0.5% DMSO (lane 2), and SCp2-Id-1E cells in GM6001 (200 μM; 0.5% DMSO) (lane 3) were plated on ECM-coated filters in Boyden chambers, and the numbers of cells that migrated through the membrane after 16 to 20 h were determined as described in Materials and Methods and the legend to Fig. 2. Error bars indicate standard deviations from three to four independent fields; the data shown are from one of three independent experiments.

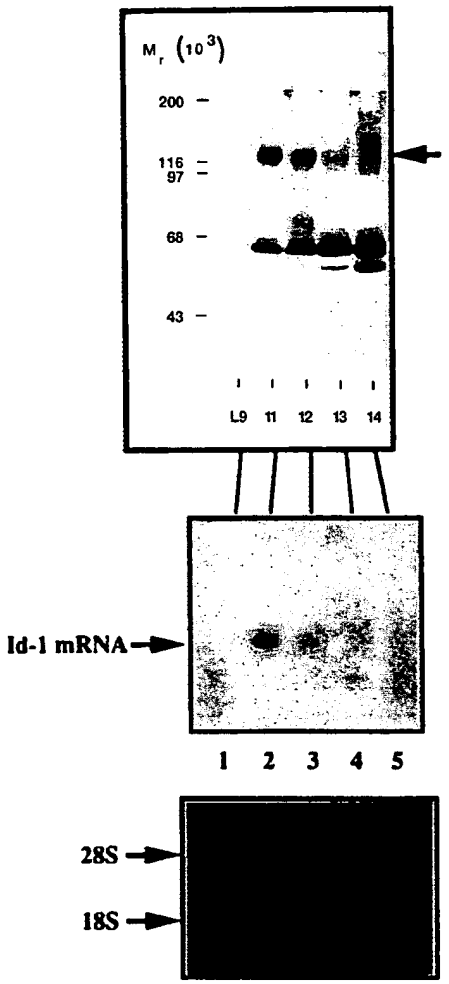


FIG. 8. Correlation between expression of the 120-kDa gelatinase and Id-1 in vivo. Cell extracts were prepared from lactating and involuting glands (as described by Talhouk et al. [41]) and analyzed by gelatin zymography. In the upper panel, the arrow marks the position of the 120-kDa gelatinase. RNA was isolated from mammary glands at the same stages and analyzed on Northern blots for Id-1 mRNA (middle panel). Lane 1, day 9 of lactation; lanes 2 through 5, days 1, 2, 3, and 4 of involution, respectively. The ethidium bromide-stained gel is shown in the lower panel to confirm RNA integrity and quantitation.

**Id-1 and the 120-kDa gelatinase are expressed during mammary gland involution.** In studying proteases during mouse mammary-gland development, Talhouk et al. (41) described a gelatinase having a molecular size greater than 110 kDa that was not expressed during lactation (Fig. 8, top panel, lane 1) but was expressed during the early stages of involution (days 1 and 2 [lanes 2 and 3], declining by day 3 [lane 4]). The identity

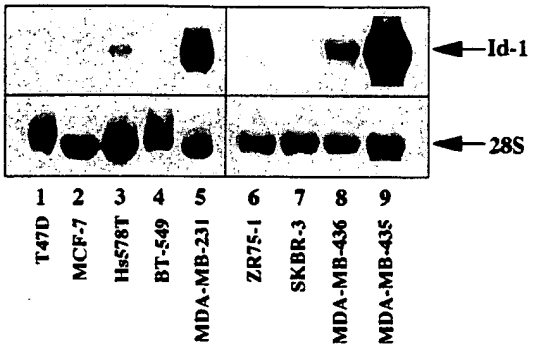


FIG. 9. Id-1 expression in nine human breast cancer cell lines. Cells were cultured in serum-free medium for 2 days before RNA was extracted and subjected to Northern blotting. The blots were then hybridized with a human Id-1 cDNA probe. Hybridization to the 28S rRNA is also indicated.

or function of this gelatinase was not determined or discussed. To explore the possibility that this gelatin-degrading proteinase may be the 120-kDa MMP expressed by Id-1-expressing cells, we isolated RNA from lactating and involuting mouse mammary glands and measured Id-1 expression by Northern analysis (Fig. 8, lower panels). Id-1 mRNA was undetectable in the lactating gland (lane 1) but was highly expressed early in involution (day 1 and 2 [lanes 2 and 3]); Id-1 expression began to decline by the 3rd day of involution (lane 4). Thus, the correlation between the expression of Id-1 and a 120-kDa gelatinase observed in mammary epithelial-cell cultures is also seen in the intact mammary gland during involution.

**Id-1 and 120-kDa gelatinase expression in invasive human breast cancer cells.** Our finding that ectopic Id-1 expression induced a 120-kDa gelatinase and an invasive phenotype in mouse mammary epithelial cells suggested that Id-1 and its associated gelatinase could, at least in some instances, contribute to human breast cancer progression. To begin to explore this possibility, we examined human breast cancer cell lines exhibiting varying degrees of invasiveness in culture and in vivo.

We examined four differentiated, essentially noninvasive breast cancer cell lines, T47D, MCF-7, ZR75-1, and SKBR-3 (43), and five poorly differentiated and invasive cell lines, Hs578T, BT-549, MDA-MB-231, MDA-MB-436, and MDA-MB-435 (27, 30, 43, 50) (Fig. 9). These cell lines have been evaluated for invasiveness in culture, by using the Boyden chamber assay (2), and in vivo, by using metastatic tumor formation in nude mice (43). By both assays (under serum-free and/or estrogen-free conditions), T47D, MCF-7, ZR75-1, and SKBR-3 cells were noninvasive. By contrast, Hs578T, BT-549, and particularly MDA-MB-231, MDA-MB-436, and MDA-MB-435 cells were highly invasive by both assays. We confirmed the reported invasive potentials of these cells, using the Boyden chamber assay (data not shown).

When cells were cultured in serum-free medium for 2 days, Id-1 mRNA was undetectable in the noninvasive T47D, MCF-7, ZR75-1, and SKBR-3 cells (Fig. 9, lanes 1, 2, 6, and 7) but was easily detectable in the highly invasive MDA-MB-231, MDA-MB-436, and MDA-MB-435 cells (Fig. 9, lanes 5, 8, and 9). Of the other invasive cells, Hs578T expressed low levels of Id-1 mRNA (Fig. 9, lane 3), whereas Id-1 mRNA was undetectable in BT-549 (Fig. 9, lane 4). Thus, the invasive potential of the human breast cancer cell lines MDA-MB-231, MDA-MB-436, MDA-MB-435, and, to a lesser extent, Hs578T could, at least in part, derive from unregulated expression of Id-1 and its associated 120-kDa gelatinase.

Consistent with this idea, a 120-kDa gelatinase was detected in conditioned media from the invasive cells that expressed Id-1 (Hs578T, MDA-MB-231, MDA-MB-436, and MDA-MB-435; Fig. 10, lanes 3, 5, 8, and 9, respectively). This gelatinase was not detected in conditioned media from the noninvasive cell lines T47D, MCF-7, ZR75-1, and SKBR-3 (Fig. 10, lanes 1, 2, 6, and 7) or from the invasive cell line that did not express Id-1 (BT-549; Fig. 10, lane 4). The 120-kDa gelatinase expressed by the human breast cancer cells comigrated with the 120-kDa gelatinase expressed by Id-1-transfected SCp2 cells (Fig. 10, lane C). As previously reported (1), the 72- and/or 92-kDa gelatinases were detected in most of these human cell lines, whether or not they were invasive. Despite the secretion of these gelatinases by the cells, only the 120-kDa gelatinase-expressing cells were invasive in the Boyden chamber invasion assay (reference 43 and data not shown). The exception was the invasive BT-549 cell line, which neither expressed Id-1 mRNA nor secreted the 120-kDa gelatinase. BT-549 cells express many MMPs (by zymography), including high levels of membrane type 1 MMPs (11).

Thus, among nine human breast tumor cells examined, only Id-1-expressing cells also expressed the 120-kDa gelatinase, and all Id-1-negative cells failed to express the 120-kDa gelatinase. Moreover, the Id-1- and 120-kDa gelatinase-expressing cells were all invasive in culture and in vivo.

## DISCUSSION

The mammary gland is one of the few organs that undergo striking morphological and functional changes during adult life, particularly during pregnancy, lactation, and involution. In both humans and mice, fetal, virgin adult, and pregnant mammary glands undergo extensive temporal and spatial remodeling, which entails invasion, migration, and relocation of cells to generate the ductal and alveolar structures of the gland. Once lactation is terminated, there is additional and extensive tissue remodeling as the gland returns to its resting state. In recent years, progress has been made in elucidating the mechanisms that regulate mammary gland-specific gene expression and the transformation of mammary epithelial cells to malignancy (3, 39). However, much less is known about the mechanisms, particularly the transcriptional mechanisms, that regulate the development and remodeling of the normal mammary gland.

**SCp2 cells as a model for normal mammary epithelial cells.** SCp2 is an immortal murine cell line that nonetheless expresses many characteristics of epithelial cells in the pregnant and lactating mammary gland. SCp2 cells proliferate in mono-

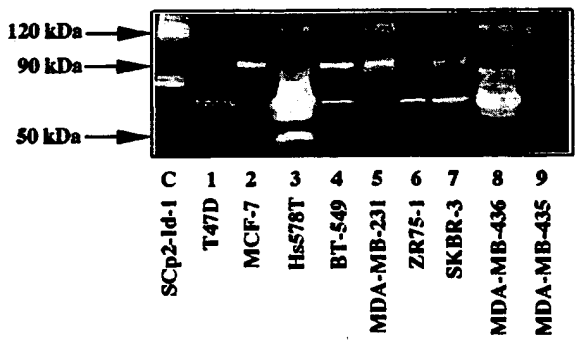


FIG. 10. Expression of a 120-kDa gelatinase in Id-1-positive cells. Serum-free conditioned media from SCp2–Id-1 transfected cells (lane C [control]) and nine human breast cancer cell lines (lanes 1 to 9) were analyzed by gelatin zymography.

layer culture in response to serum growth factors but arrest growth, form alveolar structures, and express milk proteins in response to lactogenic hormones and basement membrane components. Arrested growth is necessary, but not sufficient, for differentiation. The differentiation of SCp2 cells in culture is remarkably similar to the differentiation of mammary epithelial cells in vivo (8). Here, we extend this similarity to expression of a 120-kDa MMP that appears to be controlled by Id-1, a negative regulator of bHLH transcription factors (4).

**Id-1 as a negative regulator of mammary epithelial-cell differentiation.** During proliferation, but not during arrested growth or differentiation, SCp2 cells express Id-1. The expression of Id-1 and that of the milk protein β-casein are inversely correlated in cultured SCp2 cells (9), as well as in the mammary gland in virgin, pregnant, and lactating mice (9a). Indeed, Id-1 is a negative regulator of the functional differentiation of SCp2 cells. When constitutively expressed, Id-1 prevents the strong cell-cell contacts typical of differentiated cells and blocks milk protein expression. Although the precise mechanism by which Id-1 inhibits differentiation is not known, it is clear that it does not act by preventing the growth arrest induced by hormones and ECM (9).

Id-1 is presumed to repress differentiation by inhibiting one or more bHLH transcription factors. By analogy with the role of bHLH proteins in the differentiation of muscle, neuronal, and lymphoid cells (24, 40, 47), bHLH transcription factors may be required for differentiation-specific gene expression in the mammary gland. However, our results suggest an additional role for bHLH proteins in the mammary gland: repression of a 120-kDa MMP, whose activity permits the epithelial cells to migrate and invade the ECM.

**An Id-1-regulated gelatinase expressed by mammary epithelial cells.** Id-1 expression, whether originating from the endogenous gene or a transgene, correlated strongly with the expression of a 120-kDa gelatinase having the characteristics of an MMP. This protease appeared to be the only metalloproteinase expressed by SCp2 mammary epithelial cells. The well-characterized MMPs stromelysin and gelatinases A and B (72- and 92-kDa type IV collagenases) were not expressed by SCp2 cells. By contrast, gelatinases A and B were expressed by SCg6, a stroma-like cell line derived from the same culture from which SCp2 cells were cloned (8). SCg6 cells also express stromelysin-1 (28). These findings suggest that the expression of stromelysin and gelatinases A and B during involution of the mammary gland may derive from the nonepithelial cells in the tissue (29).

The epithelial cells of the mammary gland, on the other hand, may express the 120-kDa MMP. Talhouk et al. (41) described a gelatinase with an apparent molecular size exceeding 110 kDa that was expressed during the early stages of involution. We found that Id-1 mRNA was not expressed during lactation, when the 120-kDa gelatinase is undetectable, but was expressed early in involution (days 1 and 2). We suggest that this gelatinase may be the 120-kDa MMP identified in Id-1-expressing SCp2 cells. Thus, there is a correlation between Id-1 expression and secretion of a 120-kDa gelatinase in vivo, as well as in cultured cells.

**The Id-1-regulated gelatinase is critical for epithelial-cell invasiveness.** SCp2 cells arrest growth when in contact with basement membrane ECM. Under these conditions, Id-1 is not expressed, the cells maintain strong contacts, and they do not invade the surrounding ECM (9). Constitutive Id-1 expression did not prevent the growth arrest but conferred an invasive phenotype on the cells. Only after Id-1-expressing SCp2 cells had invaded the ECM did they resume proliferation. Thus, Id-1 appeared to be a regulator of the invasive phenotype rather than a stimulator of cell proliferation per se. This invasive phenotype, in turn, appeared to depend primarily on the 120-kDa gelatinase (MMP). This MMP was the only detectable target of GM6001, a nontoxic MMP inhibitor (12), and GM6001 effectively inhibited the invasive phenotype of Id-1-expressing cells. Thus, Id-1 and its related 120-kDa MMP were key regulators of the invasive phenotype of SCp2 cells. During involution, the Id-1-associated MMP may participate in remodeling the gland in vivo. We suggest that Id-1 and its related MMP may be key regulators of the transient invasive phenotype acquired by the epithelial cells during certain stages of normal mammary-gland development and remodeling.

**Id-1 and the 120-kDa gelatinase in tumor cell invasion.** The invasive phenotype induced by Id-1 was not the result of malignant transformation. Id-1-expressing SCp2 cells did not grow in an anchorage-independent manner and did not form detectable tumors in nude mice. Thus, Id-1 differs from oncogenes such as v-Ha-ras, which converts mouse mammary epithelial cells into invasive but also tumorigenic cells (13). Furthermore, Id-1 did not induce an invasive phenotype by converting cells to a stromal or mesenchymal phenotype. Id-1-expressing SCp2 cells maintained their epithelial characteristics, such as keratin expression, and did not express stromal MMPs. Thus, the action of Id-1 differs from that of genes of the ets family. c-Ets, a transcription factor expressed by stromal fibroblasts, promotes epithelial tumor cell invasion (48) by inducing stromal MMPs such as stromelysin-1. E1AF, a new member of the ets family, induces an invasive and migratory phenotype in human MCF-7 breast cancer cells (19), presumably by inducing gelatinase B as well as stromelysin-1.

Although the Id-1-induced invasive phenotype was not a consequence of malignant transformation, our results with human breast cancer cells suggest that constitutive Id-1 expression, and its associated 120-kDa gelatinase, may play a role in the invasive phenotype of at least some aggressive human breast tumors. We hypothesize that Id-1 and the 120-kDa gelatinase may constitute a thus far unrecognized pathway for tumor cell invasion. A very recent report (32) suggests that the Id-1–120-kDa gelatinase pathway we describe here may be of substantial clinical importance. In that report, a gelatinase of approximately the same size as the one described here was detected in urine from metastatic breast cancer patients but not in urine specimens from patients with other types of cancer. The authors acknowledge that the identity of this gelatinase is as yet unknown but suggest that it might serve as a predictor of metastatic breast cancer. By contrast, the 72- and 92-kDa gelatinases detected in urine were suggested to serve as predictors of organ-confined cancers. These suggestions are consistent with our results showing that the 72- and/or 92-kDa gelatinase is expressed by differentiated and noninvasive human breast cancer cells, whereas the 120-kDa gelatinase is expressed only in invasive breast cancer cells.

In conclusion, we propose that Id-1 regulates the invasive phenotype of breast epithelial cells, in part through the activity of a 120-kDa gelatinase, during normal mammary-gland development and remodeling. Although this phenotype is not necessarily linked to tumorigenesis, it may well be reactivated during progression toward malignancy in some breast cancers, for example, during the transition from an in situ to an invasive carcinoma. We do not yet know whether Id-1 induces the 120-kDa gelatinase by directly inactivating a bHLH repressor of the gene or whether it acts indirectly by altering the expression of other genes. We are currently attempting to clone the 120-kDa gelatinase in order to answer these questions.

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P.-Y.D. and C.Q.L. contributed equally to this work.

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## REFERENCES

1. **Abbas Abidi, S. M., E. W. Howard, J. J. Dmytryk, and J. T. Pento.** 1997. Differential influence of antiestrogens on the in vitro release of gelatinases (type IV collagenases) by invasive and non-invasive breast cancer cells. Clin. Exp. Metastasis **15**:432–439.
2. **Albini, A., Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aaronson, J. M. Kozlowski, and R. N. McEwan.** 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. **47**:3239–3245.
3. **Band, V.** 1995. Preneoplastic transformation of human mammary epithelial cells. Semin. Cancer Biol. **6**:185–192.
4. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
6. **Cross, J. C., M. L. Flannery, M. A. Blanar, E. Steingrimsson, N. A. Jenkins, N. G. Copeland, W. J. Rutter, and Z. Werb.** 1995. Hxt encodes a basic helix-loop-helix transcription factor that regulates trophoblast cell development. Development **121**:2513–2523.
7. **Danielson, K. G., C. J. Oborn, E. M. Durban, J. S. Buetel, and D. Medina.** 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc. Natl. Acad. Sci. USA **81**: 3756–3760.
8. **Desprez, P. Y., C. Roskelley, J. Campisi, and M. J. Bissell.** 1993. Isolation of functional cell lines from a mouse mammary epithelial cell strain: the importance of basement membrane and cell-cell interaction. Mol. Cell. Differ. **1**:99–110.
9. **Desprez, P. Y., E. Hara, M. J. Bissell, and J. Campisi.** 1995. Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. Mol. Cell. Biol. **15**:3398–3404.

9a. **Desprez, P. Y., C. Sympson, J. Campisi, and M. J. Bissell.** Unpublished data.

10. **Fisher, S. J., and Z. Werb.** 1995. The catabolism of extracellular matrix components, p. 261–287. *In* M. A. Haralson and J. R. Hassell (ed.), Extracellular matrix: a practical approach. IRL Press Ltd., Oxford, United Kingdom.
11. **Gilles, C., M. Polette, M. Seiki, P. Birembaut, and E. W. Thompson.** 1997. Implication of collagen type I-induced membrane type 1-matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. Lab. Investig. **76**:651–660.
12. **Grobelny, D., L. Poncz, and R. E. Galardy.** 1992. Inhibition of human skin fibroblast collagenase, thermolysin, and Pseudomonas aeruginosa elastase by peptide hydroxamic acids. Biochemistry **31**:7152–7154.
13. **Gunzburg, W. H., B. Salmons, A. Schlaeffli, S. Moritz-Legrand, W. Jones, N. H. Sarkar, and R. Ullrich.** 1988. Expression of the oncogenes mil and ras abolishes the in vivo differentiation of mammary epithelial cells. Carcinogenesis **9**:1849–1856.
14. **Hara, E., T. Yamaguchi, H. Nojima, T. Ide, J. Campisi, H. Okayama, and K. Oda.** 1994. Id-related genes encoding HLH proteins are required for G1 progression and are repressed in senescent human fibroblasts. J. Biol. Chem. **269**:2139–2145.
15. **Hara, E., J. A. Uzman, G. P. Dimri, J. O. Nehlin, A. Testori, and J. Campisi.** 1996. The helix-loop-helix Id-1 and a retinoblastoma protein binding mutant of SV40 T antigen synergize to reactivate DNA synthesis in senescent human fibroblasts. Dev. Genet. **18**:161–172.
16. **Haslam, S. Z.** 1989. The ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones. Endocrinology **125**:2766–2772.
17. **Iavarone, A., P. Garg, A. Lasorella, J. Hsu, and M. A. Israel.** 1994. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. **8**:1270–1284.
18. **Jen, Y., H. Weintraub, and R. Benezra.** 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. Genes Dev. **6**:1466–1479.
19. **Kaya, M., K. Yoshida, F. Higashino, T. Mitaka, S. Ishii, and K. Fujinaga.** 1996. A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene **12**:221–227.
20. **Kingston, R. E.** 1989. Transcription control and differentiation: the HLH family, c-myc and C/EBP. Curr. Opin. Cell Biol. **1**:1081–1087.
21. **Kjeldsen, L., A. H. Johnsen, H. Sengelov, and N. Borregaard.** 1993. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J. Biol. Chem. **268**:10425–10432.
22. **Kleiner, D. E., and W. G. Stetler-Stevenson.** 1994. Quantitative zymography: detection of picogram quantities of gelatinases. Anal. Biochem. **218**:325–329.
23. **Kreider, B. L., R. Benezra, G. Rovera, and T. Kadesch.** 1992. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science **255**:1700–1702.
24. **Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Weintraub.** 1995. Conversion of Xenopus ectoderm into neurons by neuroD, a basic helix-loop-helix protein. Science **268**:836–844.
25. **Liotta, L. A., P. S. Steeg, and W. G. Stetler-Stevenson.** 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell **64**:327–336.
26. **Lister, J., W. C. Forrester, and M. H. Baron.** 1995. Inhibition of an erythroid differentiation switch by the helix-loop-helix protein Id1. J. Biol. Chem. **270**: 17939–17946.
27. **Liu, Z., M. G. Brattain, and H. Appert.** 1997. Differential display of reticulocalbin in the highly invasive cell line, MDA-MB-435, versus the poorly invasive cell line, MCF-7. Biochem. Biophys. Res. Commun. **231**:283–289.
28. **Lochter, A., A. Srebrow, C. J. Sympson, N. Terracio, Z. Werb, and M. J. Bissell.** 1997. Misregulation of stromelysin-1 expression in mouse mammary tumor cells accompanies acquisition of stromelysin-1-dependent invasive properties. J. Biol. Chem. **272**:5007–5015.
29. **Lund, L. R., J. Romer, N. Thomasset, H. Solberg, C. Pyke, M. J. Bissell, K. Dano, and Z. Werb.** 1996. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development **122**:181–193.
30. **Maemura, M., S. K. Akiyama, V. L. Woods, Jr., and R. B. Dickson.** 1995. Expression and ligand binding of alpha 2 beta 1 integrin on breast carcinoma cells. Clin. Exp. Metastasis **13**:223–235.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Moses, M. A., D. Wiederschain, K. R. Loughlin, D. Zurakowski, C. C. Lamb, and M. R. Freeman.** 1998. Increased incidence of matrix metalloproteinases in urine of cancer patients. Cancer Res. **58**:1395–1399.
33. **Reponen, P., C. Sahlberg, P. Huhtala, T. Hurskainen, I. Thesleff, and K. Tryggvason.** 1992. Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. J. Biol. Chem. **267**:7856–7862.
34. **Reponen, P., C. Sahlberg, C. Munaut, I. Thesleff, and K. Tryggvason.** 1994. High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. J. Cell Biol. **124**:1091–1102.
35. **Riechmann, V., I. van Cruchten, and F. Sablitzky.** 1994. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucleic Acids Res. **22**:749–755.
36. **Roskelley, C. D., P. Y. Desprez, and M. J. Bissell.** 1994. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proc. Natl. Acad. Sci. USA **91**:12378–12382.
37. **Schmidhauser, C., M. J. Bissell, C. A. Myers, and G. F. Casperson.** 1990. Extracellular matrix and hormones transcriptionally regulate bovine beta-casein 5′ sequences in stably transfected mouse mammary cells. Proc. Natl. Acad. Sci. USA **87**:9118–9122.

37a. **Shalaby, R., and G. Colbern (California Pacific Medical Center).** Personal communication.

38. **Stetler-Stevenson, W. G.** 1990. Type IV collagenases in tumor invasion and metastasis. Cancer Metastasis Rev. **9**:289–303.
39. **Streuli, C. H., C. Schmidhauser, N. Bailey, P. Yurchenco, A. P. Skubitz, C. Roskelley, and M. J. Bissell.** 1995. Laminin mediates tissue-specific gene expression in mammary epithelia. J. Cell Biol. **129**:591–603.
40. **Sun, X. H.** 1994. Constitutive expression of the Id1 gene impairs mouse B cell development. Cell **79**:893–900.
41. **Talhouk, R. S., J. R. Chin, E. N. Unemori, Z. Werb, and M. J. Bissell.** 1991. Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture. Development **112**:439–449.
42. **Taub, M., Y. Wang, T. M. Szczesny, and H. K. Kleinman.** 1990. Epidermal growth factor or transforming growth factor α is required for kidney tubulogenesis in Matrigel cultures in serum free medium. Proc. Natl. Acad. Sci. USA **87**:4002–4006.
43. **Thompson, E. W., S. Paik, N. Brunner, C. L. Sommers, G. Zugmaier, R. Clarke, T. B. Shima, J. Torri, S. Donahue, M. E. Lippman, G. R. Martin, and R. B. Dickson.** 1992. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J. Cell. Physiol. **150**:534–544.
44. **Traurig, H. H.** 1967. Cell proliferation in the mammary gland during late pregnancy and lactation. Anat. Rec. **157**:489–504.
45. **Traurig, H. H.** 1967. A radioautographic study of cell proliferation in the mammary gland of the pregnant mouse. Anat. Rec. **159**:239–248.
46. **Wang, S., L. J. Counterman, and S. Z. Haslam.** 1990. Progesterone action in normal mouse mammary gland. Endocrinology **127**:2183–2189.
47. **Weintraub, H., R. Davis, S. J. Tapscott, M. Thayer, R. Krause, R. Benezra,**

**T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, et al.** 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. Science 251:761–766.

48. **Wernert, N., F. Gilles, V. Fafeur, F. Bouali, M. B. Raes, C. Pyke, T. Dupressoir, G. Seitz, B. Vandenbunder, and D. Stehelin.** 1994. Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. Cancer Res. 54: 5683–5688.
49. **Witty, J. P., J. H. Wright, and L. M. Matrisian.** 1995. Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. Mol. Biol. Cell 6:1287–1303.
50. **Zhang, R. D., I. J. Fidler, and J. E. Price.** 1991. Relative malignant potential of human breast carcinoma cell lines established from pleural effusions and a brain metastasis. Invasion Metastasis 11:204–215.

# MINA J. BISSELL
Distinguished Scientist
Lawrence Berkeley National Laboratory
One Cyclotron Rd., 83-101
Berkeley, CA 94720
mjbissell@lbl.gov


**EDUCATION**

| | | |
|---|---|---|
| Chemistry Transferred | Bryn Mawr College | 1959 – 1961 |
| B.A. (Honors) Chemistry | Radcliffe/Harvard College | 1961 – 1963 |
| M.A. Bacteriology and Biochemistry | Harvard University Medical School | 1963 – 1964 |
| Ph.D. Microbiology and Molecular Genetics | Harvard University Medical School | 1964 – 1969 |

**RESEARCH AND PROFESSIONAL EXPERIENCE:**

**Research Experience and Employment:**
Milton Fellow, Harvard Univ (1969–70); American Cancer Society Fellow (1970–72); Staff Biochemist (1972–76); Senior Staff, LBNL (1976–present); Faculty, Comparative Biochemistry (1979–present); Visiting Wellcome Prof., Kettering Inst., Univ. of Cincinnati Medical School (1986–88); Director, Cell & Molecular Biology Division, LBNL (Jan. 1988–92); Director, Life Sciences Division (includes Cell & Molecular Biology Division), LBNL (1992–2002); Distinguished Scientist (Nov. 2002-present); Senior Advisor to the Laboratory Director on Biology (Nov. 2002-present).

**Awards and Honors (selected):**
Medal for Top High School Student in the Country, Iran (1958); Medal of Amer. Inst. of Chemists for Top Chemistry Student at Radcliffe College (1962); Fogarty Senior Fellow (London, 1983–84); First Joseph Sadusk Award for Breast Cancer Research (1985); Guggenheim Fellow (Paris, 1992-93); ASCB Women in Cell Biology Career Recognition Award (1993); Elected AAAS Fellow (1994); E.O. Lawrence Award, US Dept. of Energy (1996); President, American Society of Cell Biology (ASCB,1997); Elected, Inst. of Medicine of the National Acad. of Sciences (1997); Exceptional Service Award, OBER, US Dept. of Energy (1997); Mellon Award, University of Pittsburgh (1998); Eli Lily/Clowes Award of the American Association for Cancer Research (AACR;1999); President, Int'l Society of Differentiation (ISD; 2000–2002); Honorary Doctorate, Pierre & Marie Curie University, Paris, France (2001); Innovator Award in Breast Cancer, US Department of Defense (2002); Elected to the American Academy of Arts and Sciences (2002); Komen Foundation Brinker Award (2003); More than 40 distinguished & named lectures.

**National & International Committees and Review Boards (selected):**
NIH Molecular Cytology Study Section (1981-85); NIH Gerontology & Geriatrics Review Study Section (1987–89); NIH Pathology B Study Section (1989–92); Board of Directors, Gordon Conferences (1993–98); Chair, Gordon Research Conference, Biological Structure and Gene Expression (1993); Secretary of Energy's Advisory Committee BERAC (1995–1999); Chair, BERAC Subcommittee on Application of Genome and Structural Biology (1995); Chair, Keystone Meeting on Breast and Prostate Cancer, Taos, NM (1996); Chair, NASA Committee on the Role of Animal Research in Space (1996–97); Integration Panel, U.S. Army Breast Cancer Research Program (1995–2003); NIH and NCI Panel on "Preclinical Models of Cancer" (1997-98); Howard Hughes Medical Inst. Evaluation Panel, Washington, DC (1997/1999); Board of Directors, American Association for Cancer Research (1999–2001);U.S. Representative to Council of Scientists, Human Frontier Science Program, Strasbourg, France (1998–2002); Advisory Committee, Burroughs Wellcome Fund's Career Awards in the Biomedical Sciences program (1998–2002); Human Rights Committee of National Academy of Sciences (1999–present); Advisory Board, Univ. Chicago Cancer Research Center (1998–present); Institute of Defense Analysis, Defense Science Study Group, Alexandria, VA (2000–present); AACR Science Policy and Legislative Affairs Committee (2001–2004); External Advisory Board, Institute for Molecular and Cell Biology, Porto, Portugal (IBMC) (1999–present); External Scientific Advisory Committee for the MIT Center for Environmental Health Sciences (2002–present.); Science Advisory Committee, Breakthrough Breast Cancer, London, UK (2002-present).

**Associate Editor & Editorial Boards (current only):**
*In Vitro Cellular and Developmental Biology* (1990– ); *Journal of Cellular Biochemistry* (1990– ); *Molecular Carcinogenesis* (1993– ); *Cancer Research* (1994–; Senior Assoc. Editor, 2000- ); *The Breast Journal* (1994– ); *Cell Structure and Function* (1994– ); *Journal of Mammary Gland Biology* (1995– ); *Journal of Experimental Therapeutics* and Oncology (1995– ); *Molecular Medicine* (1997– ); *Breast Cancer Research* (1999– ; Senior Editor: 2003- ); *International Journal of Cancer* (1999– ); *The FASEB Journal* (2002– ).

**Patent Pending:**
IB–JIB-1791 A Suprabasal Breast Cell Line with Stem Cell Properties (with Olé William Petersen et al.)

**Patents Issued:**
United States Patent # 5,846,536: Restoration of Normal Phenotype in Cancer Cells
United States Patent # 6,123,941: Method for Restoration of Normal Phenotype in Cancer Cells
United States Patent # 6,287,790: Utilization of Nuclear Structural Proteins for Targeted Therapy and Detection of Proliferative and Differentiation Disorders

**Lectures (2002-Present):** *Plenary and named lectures are marked with an asterisk.*

**2002**
*Keystone Symposium, Biological Response to the Extracellular Matrix, The Matrix in Development, Banff, Canada
*Excellence in Cancer Research Seminar Series, Cross Cancer Institute, Edmonton, Alberta, Canada
*Bioengineering and Environmental Health & Chemistry Department, MIT, Boston, MA (Harris Lecture)
*Matrix and Morphogenesis: Celebrating Elizabeth Hay, Boston, MA
*International Society of Differentiation, Lyon, France (President's Lecture)
*DOD, Era of Hope, Breast Cancer Research Program Meeting, Orlando, FL (Innovation Lecture)
*Van Andel Institute, Grand Rapids, MI
*AACR (Proteases, Extracellular Matrix, and Cancer) Hilton Head, SC (Keynote Lecture)
*International Biomed Society, Stanford, CA

**Others include**: City of Hope, Los Angeles, CA; UCSF, Department of Surgery, San Francisco, CA; University of California, Los Angeles, Molecular Biology Institute, Graduate Program Seminar, Los Angeles, CA; Thomas Jefferson University, Kimmel Cancer Center, Philadelphia, PA; Duke University Medical Center, Signal Transduction Colloquium, Durham, NC Tufts University Medical School, Department of Anatomy and Cellular Biology, Boston, MA; Boston University, Boston, MA Biogen, Cambridge, MA; Morehouse College, Division of Science and Mathematics, Atlanta, GA; Emory University, Atlanta, Department of Cell Biology, GA; Whitehead Institute, Cambridge, MA; Harvard Medical School, Vascular Biology Seminar, Department of Pathology, Boston, MA; University of California, Davis, Medical Cancer Center, Sacramento, CA Oakland Outreach Program, Oakland, CA; Breast Cancer Foundation's Think Tank, Santa Barbara, CA; AstraZeneca, Boston, MA; Becton Technologies, RTP, North Carolina

**2003**

*Second Timberline Symposium on Epithelial Biology, My Hood, Oregon
*American Society of Investigative Pathology Conference, San Diego, CA
*Northwestern University, Chicago, IL (Mayberry Lecture)
*Danish Society for Cancer Research, Copenhagen, Denmark, Bristol (Myers Squibb Lecture)
*Gordon Research Conference: Mammary Gland Meeting, Bristol RI
*Gordon Research Conference: Cell Contact and Adhesion, Andover, NH (Keynote session)
*The Cancer Institute of New Jersey, Princeton, NJ
*University of Illinois, in Chicago, Chicago, IL (Krakower Lecture)
*Mahajani Symposium, San Diego, CA
*Epithelial-Mesenchymal Transitions Conference, Queensland, Australia
*University of California, Berkeley (BISC Distinguished Lecture)
*San Antonio Breast Cancer Conference (Komen Foundation, Brinker Award)

**Others include**: Caltech, Pasadena, CA; UCSF, Cancer Center, San Francisco, CA; Salk Institute, San Diego, CA University of California, San Diego, CA; University of California, Santa Cruz, CA; Huntsman Cancer Institute, Salt Lake City, Utah; Endocrine Therapy Conference, Boston, MA; Tularik Inc. San Francisco, CA; Ernest Gallo Clinic and Research Center, Emeryville, CA; Cell Genesys, San Francisco, CA; California Breast Cancer Research Program, San Diego, CA ComBio 2003 Meeting, Melbourne, Australia.

**Publications (selected since 1995; total 236):**


131. Boudreau N, Sympson CJ, Werb Z and **Bissell MJ**. (1995) Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 267:891-893.
135. Lin CQ, Dempsey P, Coffey C and **Bissell MJ**. (1995) Extracellular matrix regulates whey acidic protein gene expression by suppression of TGF-a in mouse mammary epithelial cells: Studies in culture and in transgenic mice. *J. Cell Biol* 129(4):1115-1126.
137. Rønnov-Jessen L, Petersen OW, Kotelianski V and **Bissell MJ**. (1995) The origin of the myofibroblasts in breast cancer: Recapitulation of tumor environment in culture unravels diversity and implicates coverted fibroblasts and recruited smooth muscle cells. *J Clinical Investigation* 95:859-873.
139. Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz A and **Bissell MJ**. (1995) Laminin mediates tissue-specific gene expression in mammary epithelia. *J Cell Biol* 129:591-603.
154. Rønnov-Jessen L, Petersen OW and **Bissell MJ**. (1996) Cellular changes involved in conversion of normal to malignant breast: The importance of the stromal reaction. *Physiol Revs* 76:69-125.
159. Ashkenas J, Muschler J and **Bissell MJ**. (1997) The extracellular matrix in epithelial biology: Shared molecules and common themes in distant phyla. *Developmental Biol* 180:433-444.
162. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z and **Bissell MJ**. (1997) Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 139:1861-1872.
163. Lochter A, Srebrow A, Sympson CJ, Terracio N, Werb Z and **Bissell MJ**. (1997) Misregulation of stromelysin-1 expression in mouse mammary tumor cells accompanies acquisition of stromelysin-1-dependent invasive properties. *J Biol Chem* 272:5007-5015.
165. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C and **Bissell MJ**. (1997) Reversion of the malignant phenotype of human breast cells in three-dimensional culture and *in vivo* using integrin blocking antibodies. *J Cell Biol* 137:231-246 (cover feature).
171. Hirai Y, Lochter A, Galosy S, Koshida S, and **Bissell MJ**. (1998) Epimorphin, not hepatocyte growth factor or epidermal growth factor, functions as a morphoregulatory molecule for mammary epithelial cells. *J Cell Biol* 140:159-169.
172. Lelièvre SA, Weaver VM, Nickerson JA, Larabell CA, Bhaumik A, Petersen OW and MJ **Bissell MJ**. (1998) Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus. *Proc Natl Acad Sci USA* 95:14711-14716.
175. Myers CA, Schmidhauser C, Mellentin-Michelotti J, Fragoso G, Roskelley CD, Casperson G, Mossi R, Pujuguet P, Hager G and **Bissell MJ**. (1998) Characterization of BCE-1: A transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. *Mol Cell Biol* 18(4):2184-2195.
180. Wang F, Weaver VM, Petersen OW, Larabell CA, Dedhar S, Briand P, Lupu R and **Bissell MJ**. (1998) Reciprocal interactions between β1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology. *Proc Natl Acad Sci USA* 95:14821-14826.
182. **Bissell MJ**. (1999) Tumor plasticity allows vasculogenic mimicry, a novel form of angiogenic switch: A rose by any other name? *Am J Pathol* 155(3):675-9.
184. **Bissell MJ**, Weaver VM, Lelièvre SA, Wang F, Petersen OW and Schmeichel KL. (1999) Tissue structure, nuclear organization and gene expression in normal and malignant breast. *Cancer Res.* 59:1757s-1764s.
185. Lochter A, Navre M, Werb Z and **Bissell MJ**. (1999) β1 and β2 integrins mediate invasive activity of mouse mammary carcinoma cells through regulation of stromelysin-1 expression. *Mol Biol Cell* 10:271-282.
188. Muschler J, Lochter A, Roskelley CR, Yurchenco P and **Bissell MJ**. (1999) Division of labor among the α6ß4 integrins and an E3 laminin receptor to signal morphogenesis and ß-casein expression in mammary epithelial cells. *Mol Biol Cell* 10(9):2817-28.
189. Péchoux C, Gudjonsson T, Rønnov-Jessen L, **Bissell MJ** and Petersen OW. (1999) Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. *Develop Biol* 206:88-99.
191. Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier J-P, Gray J, Pinkel D, **Bissell MJ**, and Werb Z. (1999) The stromal proteinase MMP-3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98(2):137-146.
195. Pujuguet P, Simian M, Liaw J, Timpl R, Werb Z and **Bissell MJ**. (2000) Nidogen-1 regulates laminin-1-dependent mammary-specific gene expression. *J Cell Sci* 113 (Pt 5):849-858 (cover feature).
197. Chen M, Schmeichel K, Mian IS, Lelièvre SA, Petersen OW and **Bissell MJ**. (2000) AZU-1: A candidate breast tumor suppressor and biomarker for tumorigenic reversion. *Mol Biol Cell* 11(4):1357-1367.

200. Hirai Y, Radisky D, Boudreau R, Simian M, Stevens M, Oka Y, Takebe K, Niwa S and **Bissell MJ**. (2001) Epimorphin mediates mammary luminal morphogenesis through control of C/EBPβ. *J Cell Biol* 153(4):785-794.
201. Simian M, Hirai Y, Navre M, Werb Z, Lochter A and **Bissell MJ**. (2001) The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development* 28:3117-3131.
202. Muthuswamy SK, Li D, Lelièvre SA, **Bissell MJ** and Brugge JS. (2001) Erbβ2, but not Erbβ1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nature Cell Biol* 3(9):785-792.
203. **Bissell MJ** and Radisky D. Putting tumours in context. (2001) *Nature Reviews (Cancer)* 1:48-54.
208. Gudjonsson T, Rønnov-Jessen L, Villadsen R, **Bissell MJ** and Petersen OW. (2002) Normal and tumor-derived myoepithelial cells differ in their ability to signal to luminal breast epithelial cells for polarity and basement membrane deposition. *J Cell Science* 115(1):39-50.
211. Gudjonsson T, Villadsen R, Nielsen HL, Rønnov-Jensen L, **Bissell MJ**, and Petersen OW. (2002) Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. *Genes & Development* 16(6):693-706.
213. Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA and **Bissell MJ**. (2002) Phenotypic Reversion or Death of Cancer Cells by Altering Signaling Pathways in Three-Dimensional Contexts. *J Nat'l Cancer Inst* 94(19):1494-1503.
214. Weaver VM, Lelièvre SA, Lakins JN, Chrenek MA, Jones JCR, Giancotti F, Werb Z and **Bissell MJ**. (2002) β4 Integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. *Cancer Cell* 2:205-216; ***News & Views, Nature 419:790-791 (2002). Minireview (Cell) 111:923-925* (2002).**
215. Muschler J, Levy D, Boudreau R, Henry M, Campbell K and **Bissell MJ**. (2002) A role for dystroglycan in epithelial polarization: Loss of function in breast tumor cells. *Cancer Research* 62:7102-7109.
216. **Bissell MJ**, Radisky D, Rizki A, Weaver, VM and Petersen, OW. (2002) The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation* 70(9-10):537-46.
217. Petersen OW, Nielsen HL, Gudjonsson T, Villadsen R, Rank F, Niebuhr E, **Bissell MJ** and Rønnov-Jessen L. (2003) Epithelial to Mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol* 162:2:391-402.
218. **Bissell MJ**, Mian IS, Radisky D and Turley E. (2003) Tissue-specificity: Structural cues allow diverse phenotypes from a constant genotype. In: Origination of Organismal Form: Beyond the Gene in Developmental and Evolutionary Biology, Müller GB and Newman SA (Eds.). The Vienna Series in Theoretical Biology. *MIT Press* 7:103-117.
219. **Bissell MJ** and Bilder D. (2003) COMMENTARY: Polarity determination in breast tissue: desmosomal adhesion, myoepithelial cells, and laminin 1. *Breast Cancer Res* 5:2:117-119.
220. Anders M, Hansen R, Ding R-X, Waldman FW, Rauen K, **Bissell MJ**, and Korn WM. (2003) Disruption of 3D tissue integrity facilitates adenovirus infection by deregulating the coxsackievirus and adenovirus receptor. *Proc Natl Acad Sci* 100:4:1943-1948.
221. Bhatacharyya C, Grate LR, Rizki A, Radisky D, Molina FJ, Jordan MI, **Bissell MJ**, Mian IS. (2003) Simultaneous relevant feature identification and classification in high-dimensional spaces: Application to molecular profiling data. *Signal Processing* 83:4:729-743.
222. Schmeichel, KL and **Bissell MJ**. (2003) Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci.* 116:2377-2388.
223. Novaro V, Roskelley C and **Bissell MJ**. (2003) Collagen-IV or laminin-1 together with lactogenic hormones regulate estrogen receptor-alpha expression and function in mouse mammary epithelial cells. *J Cell Science* 116(14) 2975-2986.
224. Gudjonsson T, Ronnov-Jessen L, Villadsen R, **Bissell MJ**, Petersen OW. (2003) To create the correct microenvironment: three-dimensional heterotypic collagen assays for human breast epithelial morphogenesis and neoplasia. *Methods* Jul;30(3):247-55.
225. Radisky, DC, Hirai, Y and **Bissell MJ.** (2003) Delivering the message: epimorphin and mammary epithelial morphogenesis. *Trends Cell Biol* 13(8):426-34.
226. Park, CC, Henshall-Powell, RL, Erickson, AC, Talhouk, R, Parvin, B, **Bissell MJ** and Barcellos-Hoff MH. Ionizing radiation induces heritable disruption of epithelial cell interactions. *Proc Natl Acad Sci* 100:19:10728-10733.
227. Wiseman BS, Sternlicht MD, Lund LR, Alexander CM, Mott J, **Bissell MJ**, Soloway P, Itohara S and Werb Z. (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *Journal of Cell Biology* 162 (6):1123-1133.
228. Fata JE, Werb, Z and **Bissell MJ.** (2003) Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res* Review 6:1-11.
229. Petersen OW, Gudjonsson T, Villadsen R, **Bissell MJ**, Ronnov-Jessen L. (2003) Epithelial progenitor cell lines as models of normal breast morphogenesis and neoplasia. *Cell Prolif.* Oct;36 Suppl 1:33-44. Review.
230. Park C, Zhang H, Peng M, **Bissell MJ**. (2003) Cell-ECM mediated radiation response in breast cancer: beta1 integrin as a potential molecular target. *Int J Radiat Oncol Biol Phy* Oct 1;57(2 Suppl):S161.
231. Kenny, PA and **Bissell MJ**. (2003) Tumor reversion: Correction of malignant behavior by microenvironmental cues. *Int J Cancer* Review 107(5):588-695.
232. **Bissell MJ**, Rizki A, Mian IS (2003) Tissue architecture: the ultimate regulator of breast epithelial function. *Curr Opin Cell Biol* Dec;15(6):753-62.
233. Come SE, Buzdar AU, Arteaga CL, **Bissell MJ**, Brown MA, Ellis MJ, Goss PE, Green JE, Ingle JN, Lee AV, Medina D, Nicholson RI, Santen RJ, Schiff R, Hart CS. Proceedings of the third international conference on recent advances and future directions in endocrine manipulation of breast cancer: conference summary statement. *Clin Cancer Re.* 2004 Jan 1;10(1 Pt 2):327S-30S.
234. Novaro V, Radisky DC, Ramos Castro NE, Weisz A, **Bissell MJ**. (2004) Malignant mammary cells acquire independence from extracellular context for regulation of estrogen receptor alpha. *Clin Cancer Res* Jan 1;10(1 Pt 2):402S-9S.
235. Boudreau N, Myers C, **Bissell MJ**. (2004) From laminin to lamin: regulation of tissue-specific gene expression by the ECM. *Trends Cell Biol* 1995 Jan;5(1):1-4.
236. Liu H, Radisky DC, Wang F and **Bissell MJ**. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. *J Cell Biol* (In press -2004).

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

| NAME | POSITION TITLE |
|---|---|
| Muschler, John L. | Associate Scientist |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| University of Illinois, Urbana, IL | B.S. | 1986 | Chemistry |
| University of Illinois, Urbana, IL | Ph.D. | 1993 | Biochemistry |

**Professional Experience:**

1984-85 **Research Associate**, U.S.D.A. laboratory, University of Illinois, Urbana.
1986-87. **Research Associate**, Department of Biochemistry, University of Illinois.
1987 **Teaching Assistant**, Chemistry, University of Illinois, Urbana.
1989 **Teaching Assistant**, Biochemistry, University of Illinois, Urbana.
1993-1995 **Postdoctoral Research Fellow**
Laboratoire de Biologie Moleculaire,
Pasteur Institute, Paris, France
1995-2000 **Postdoctoral Research Fellow**
Life Science Division
Lawrence Berkeley National Laboratory, Berkeley, CA.
2000-2002 **Scientist**
Life Science Division,
Lawrence Berkeley National Laboratory, Berkeley, CA.
2002-present **Associate Scientist**
California Pacific Medical Center Research Institute,
San Francisco, CA.

2003 Grant Reviewer for the Department of Defense Breast Cancer Research Program.

**Honors and Fellowships:**

1989 University of Illinois Biotechnology Center Award.
1993-1995 Long Term Fellowship, European Molecular Biology Organization (EMBO).
1995-1997 Individual National Research Service Award.
1998-2000 Postdoctoral Fellowship, Department of Defense Breast Cancer Research Program.
2000-2003 New Investigator Award, California Breast Cancer Research Program.
2001-2004 IDEA Grant, Department of Defense Breast Cancer Research Program.

**Publications:**

Henry, I., Forlani, S., Vaillant, S., Muschler, J., Choulika, A. and Nicolas, J.-F. (1999) "LagoZ and LagZ, two genes derived from LacZ by depletion of CpG dinucleotides for the study of epigenetic controls". C. R. Acad. Sci., Paris. 322: 1-9.

Muschler, J., Lochter, A., Roskelley, C.D., Yurchenco, P. and Bissell, M.J. (1999). Division of labor among the α6β4 integrin, β1 integrins, and an E3 laminin receptor to signal morphogenesis and β-casein expression in mammary epithelial cells. Mol. Biol. Cell 10: 2817-2828.

McDonald KA., Muschler J., and Horwitz AF. (1998). Immunopurification of a sarcomeric junctional protein complex containing GAPDH. Exp. Cell Res. 243: 305-18

Lochter A., Galosy S., Muschler J., Freedman N., Werb Z. and Bissell MJ. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J. Cell Biol. 139:1861-72

Ashkenas J., Muschler J. and Bissell MJ. (1996). The extracellular matrix in epithelial biology: shared molecules and common themes in distant phyla. Dev. Biol. 180:433-44

Sastry SK., Lakonishok M., Thomas DA., Muschler J. and Horwitz AF. (1996). Integrin alpha subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. J. Cell Biol. 133:169-84

Lakonishok M., Muschler J. and Horwitz AF. (1992). The α5β1 integrin associates with a dystrophin-containing lattice during muscle development. Dev. Biol. 152:209-20

Bronner-Fraser M., Artinger M., Muschler J. and Horwitz AF. (1992). Developmentally regulated expression of α6 integrin in avian embryos. Development 115:197-211

Bao ZZ., Muschler J. and Horwitz AF. (1992). LBL, a novel, developmentally regulated, laminin-binding lectin. J. Biol. Chem. 267:4974-80

Muschler JL. and Horwitz AF. (1991). Down-regulation of the chicken α5β1 integrin fibronectin receptor during development. Development 113:327-37

**Recent Abstracts:**

Weir, L., Itahana, Y., Campbell, K., Bissell, M.J., Muschler, J. Dystroglycan is essential for the response of mammary epithelial cells to laminin-1 and HSPGs. American Society for Cell Biology Annual Meeting. (Dec., 2003). San Francisco, CA.

Singh, J, Itahana, Y, Campbell, K., Muschler J. Proteolytic enzymes and altered glycosylation modulate dystroglycan function in carcinoma cells. American Society for Cell Biology Annual Meeting. (Dec., 2003). San Francisco, CA.

Mott, J., Muschler, J., Bissell, M.J. Phenotypic Reversion of the Human Breast Tumor Cell Line T4-2 Correlates with a Decrease in MMP-9 Production. American Society for Cell Biology Annual Meeting. (Dec., 2003). San Francisco, CA.

Muschler, J., Campbell, K. and Bissell, M.J. Restoration of dystroglycan function as a novel target in breast cancer therapy". The Era of Hope Meeting of the Department of Defense Beast Cancer Research Program. (Sept., 2002). Orlando, FL.

Muschler, J., Levy, D., Henry, M., Campbell, K. and Bissell, M.J. Loss of dystroglycan in breast tumor cells. American Society for Cell Biology Annual Meeting. (Dec., 2001). Washington D.C.

**Invited Lectures and Symposia:**

"Restoration of DG Function as a novel target in breast cancer therapy". The Era of Hope Meeting of the Department of Defense Beast Cancer Research Program. (Sept., 2002). Orlando, FL. 2002

"Linking dystroglycan, polarity and tumor suppression in breast epithelial cells." University of California at San